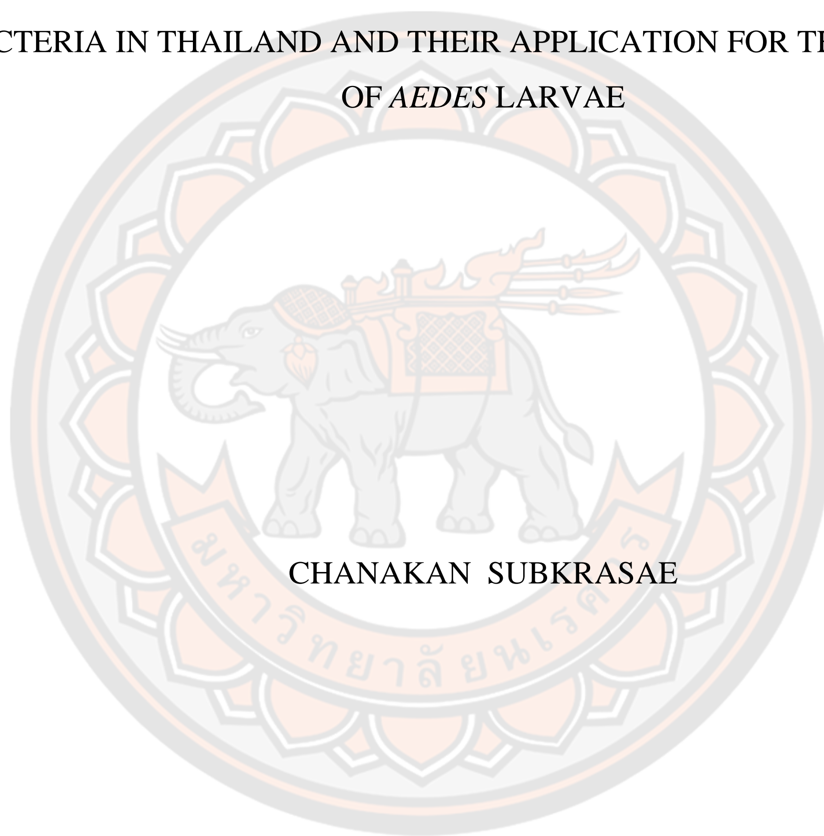
DIVERSITY OF ENTOMOPATHOGENIC NEMATODES AND SYMBIOTIC BACTERIA IN THAILAND AND THEIR APPLICATION FOR THE CONTROL OF *AEDES* LARVAE



CHANAKAN SUBKRASAE

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Doctor of Philosophy in Parasitology
2023

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Thesis entitled "Diversity of entomopathogenic nematodes and symbiotic bacteria in Thailand and their application for the control of *Aedes* larvae"
By Chanakan Subkrasae
has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy in Parasitology of Naresuan University

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ABSTRACT

Aedes mosquitoes, especially *Aedes aegypti* and *Aedes albopictus* are important vectors for several arboviruses such as the dengue virus. The chemical control of *Aedes* spp., which is usually implemented, affects both humans and the environment. Moreover, *Ae. aegypti* resistance to chemical insecticides has been reported worldwide. To tackle this, entomopathogenic nematodes (EPNs) and their symbiotic bacteria (*Photorhabdus* and *Xenorhabdus*) may be an alternative bio-control agent that can overcome such issues. Thus, this study aims to isolate, identify, and analyze the phylogeny of EPNs and their symbiotic bacteria in Thailand and evaluate their efficacy in controlling the *Aedes* larvae. From 12 provinces in Thailand, soil samples were randomly collected, with 118 out of 1,100 them being positive for EPNs (10.73% prevalence) in genera *Steinernema* (4.46%) and *Heterorhabditis* (6.27%). Then, molecular discrimination of these two genera was performed based on the sequencing and phylogenetic analysis of the 28S rDNA and internal transcribed spacer regions (ITS). The most abundant species of EPN were *Heterorhabditis indica*, with minor species of *Heterorhabditis* sp. SGmg3, *H. baujardi*, *S. surkhetense*, *S. kushidai*, *S. siamkayai,* *Steinernema* sp.

YNd80, *Steinernema* sp. YNc215, *S. guangdongense*, and *S. huense*. Symbiotic bacteria were isolated from the EPNs and identified based on the colony morphology as *Photorhabdus* (69 isolates) and *Xenorhabdus* (49 isolates). The molecular identification of symbiotic bacteria with *recA* sequencing indicated that most were *P. luminescens* subsp. *akhurstii* and *X. stockiae* with minor prevalence of *P. luminescens* subsp. *hainanensis*, *P. asymbiotica* subsp. *australis*, *X. indica*, *X. griffiniae*, *X. japonica*, *X. thuongxuanensis*, and *X. eapokensis*. The larvicidal activity of five selected EPN isolates were tested against *Ae. aegypti*. Ten larvae of *Ae. aegypti* were incubated with different concentrations (80, 160, 320, and 640 IJs/larva) of the infective juveniles of EPN in 24-well and 6-well plates for 4 days. The mortality rates of the larvae were observed daily. *Steinernema surkhetense* (ePYO8.5_TH) showed the potential to kill *Ae. aegypti* larvae, with the highest mortality rate of 92 ± 9.37% and 89 ± 9.91% after it was treated with 640 IJs/larva in a 24-well plate and 1600 IJs/larva in a 6-well plate, respectively. The larvicidal bioassays of symbiotic bacteria were also tested with *Ae. aegypti* and *Ae. albopictus* larvae. The results suggested that a whole-cell suspension of *X. griffiniae* (bMSN3.3_TH) had the highest efficiency in eradicating *Ae. aegypti* and *A. albopictus*, with 90 ± 3.71% and 81 ± 2.13% mortality, respectively, after 96 h exposure. In contrast, 1% of ethyl acetate extracted from *X. indica* (bSNK8.5_TH) showed reduced mortality for *Ae. aegypti* of only 50 ± 3.66% after 96 h exposure. In conclusion, this study revealed an abundant distribution of EPNs and their symbiotic bacteria across Thailand, and *S. surkhetense* (ePYO8.5_TH), *X. griffiniae* (bMSN3.3_TH) and *X. indica* (bSNK8.5_TH) may be used as a biocontrol agent against *Aedes* larvae.

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Chanakan Subkrasae

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CHAPTER I

INTRODUCTION

Background and significance of the study

Aedes spp. are the mosquito in order Diptera and are vector for several viruses such as Japanese encephalitis, West Nile, Chikungunya, and dengue viruses (Martinet, Ferté, Failloux, Schaffner, & Depaquit, 2019). Recently, *Aedes* mosquitoes are reported to be a vector for Zika virus which was considered a major public health threat worldwide (Benelli, & Mehlhorn, 2016; Gebre, Forbes, & Gebre, 2016). In addition, *Aedes* spp. are hosted by filarial worms which is the cause of elephantiasis in human (Gleave, Cook, Taylor, & Reimer, 2016). *Aedes aegypti* and *Aedes albopictus* are recognized as the main vectors for dengue virus, which causes hemorrhage fever in humans (Bhatt et al., 2013; World Health Organization, 2020a). In the year 2017, 500,000 cases were reported with severe dengue occurred worldwide as estimated by the World Health Organization (WHO), and 2.5% of these patients died (World Health Organization, 2020a). In Thailand, dengue fever (Dengue fever: DF, Dengue hemorrhagic fever: DHF, Dengue shock syndrome: DSS) is the most common mosquito-borne diseases that made 100,000 patients with a hundred deaths in 2019 (Bureau of Epidemiology, 2019a, 2019b, 2019c)

Control of *Aedes* are necessary to be implement while the development of vaccine and effective drug for treatment of those diseases are still under progress. In general, chemical control for *Aedes* based on organophosphates and organochlorines are commonly used as it is high efficacy and rapid effective on both adult and larval mosquitoes. However, the repeated use of the chemicals leads to emerge of chemical-resistant mosquitoes (Elia-Amira et al., 2018; Moyes et al., 2017). In addition, accumulated insecticidal chemicals are toxic to animal and human health (Ansari, Moraiet, & Ahmad, 2014). Therefore, biological control of *Aedes* is alternative method to overcome these problems. Entomopathogenic nematodes (EPNs) associated with symbiotic bacteria (*Xenorhabdus* and *Photorhabdus*) have been reported as the biocontrol agents for several insect pests (Georgis et al., 2006).

Entomopathogenic nematodes in genus *Steinernema* and *Heterorhabditis* are symbiotic associated with bacteria in genus *Xenorhabdus* and *Photorhabdus*, respectively. The nematode-bacterium complex causes insect larvae death within 24-48 h via their secondary metabolites (Forst, Dowds, Boemare, & Stackebrandt, 1997; Goodrich-Blair, & Clarke, 2007). *Steinernema* spp. have been used to control sugar froghopper (*Aeneolaimia varia*), ground pearl scale (*Eurhizococcus colombianus),* and spittlebug (*Mahanarva spectabilis*) while *Heterorhabditis* have been used to control fall armyworm (*Spodoptera frugiperda*), coffee root mealybug (*Dysmicoccus texensis*) and Mexican bean beetle (*Copturus aguacatae*) (Andaló et al., 2012; Batista, Auad, Andaló, & Monteiro, 2014). In addition, *Xenorhabus* and *Photorhabdus* bacteria were reported to be pathogenic agent to *Aedes* spp. (Fukruksa et al., 2017; Vitta et al., 2018).

Entomopathogenic nematodes and their symbiotic bacteria can be found across various environments worldwide (Vitta et al., 2017). At present, one hundred species of *Steinernema* and 28 species of *Heterorhabditis* have been reported together with 26 species of *Xenorhabdus* and 5 species of *Photorhabdus* were also documented. However, six species of *Steinernema (S. abbasi, S. khoisanae, S. kushidoi, S. scarabaei, S. seungi*, and *S. websteri)*, 7 species of *Heterorhabditis (H. bacteriophora, H. baujardi, H. gerrardi, H. indica, H. zaelandica, Heterorhabditis* sp. SGgj, and *Heterorhabditis* sp. SGmg3), 6 species of *Xenorhabdus (X. ehlersii, X. ishibashii, X. japonica, X. miraniensis, X. stockiae*, and *X. vietnamensis)* and 3 species with 5 subspecies of *Photorhabdus (P. luminescens* subsp. *akhurstii, P. luminescens* subsp. *laumondii, P. luminescens* subsp. *hainanensis, P. temperata* subsp. *temperate*, and *P. asymbiotica* subsp. *australis)* were recorded in Thailand. Thus, the objectives of the present study were to isolate and identify EPNs and their symbiotic bacteria in Thailand and evaluate their efficacies on *Aedes* larvae.

Purposes of the study

1. To isolate and identify EPNs from soil samples in Thailand
2. To isolate and identify *Photorhabdus* and *Xenorhabdus* bacteria associated with EPNs
3. To study phylogenetic trees of EPNs and their symbiotic bacteria

4. To determine efficiency of EPNs and symbiotic bacteria against *Aedes* larvae

Scope of the study

In this study, a thousand of the soil samples from 200 soil sites was randomly collected in the diverse areas in 12 provinces of Thailand (Chiang Mai, Kalasin, Khon Kaen, Lopburi, Mae Hong Son, Nan, Phayao, Phetchaburi, Phrae, Sakon Nakhon, Saraburi and Uttaradit. The infective juvenile stage (IJ) of EPN in the soil samples was isolated using *Galleria mononella* larvae as bait. White trap technique was performed to isolate the IJ from the insect cadavers. Molecular techniques based on sequencing of internal transcribe spacer for *Heterorhabditis* and 28S rDNA for *Steinernema* were used to identify the EPNs. A partial sequence of the *recA* gene was analyzed to identify their symbiotic bacteria. In addition, phylogenetic tree was analyzed based on neighbor joining and maximum likelihood methods using MEGA program. Larvicidal activity of EPNs and their symbiotic bacteria against *Aedes agypti* and *Aedes albopictus* was evaluated in the laboratory.

Keywords

Entomopathogenic nematode, *Steinernema*, *Heterorhabditis*, *Photorhabdus*, *Xenorhabdus*, *Aedes*, Biological control

CHAPTER II

RELATED WORKS AND STUDIES

Entomopathogenic nematodes

Entomopathogenic nematodes (EPNs) belonging to genus *Steinernema* and *Heterorhabditis* are mortal parasites of several insects (Ramos-Rodríguez, Campbell, & Ramaswamy, 2007). EPNs have been widely used as biological control agents of insect pests (Andaló et al., 2012; Aristizábal et al., 2015; Batista et al., 2014). The United States Environmental Protection Agency (EPA) has confirmed that the EPNs are harmless for human, vertebrates and plants (Kaya, & Gaugler, 1993). Entomopathogenic nematodes are classified in family Heterorhabditidae (genus *Heterorhabditis*) and Steinernematidae (genus *Steinernema*). They are mutually symbiosis with bacteria in genus *Photorhabdus* and genus *Xenorhabdus*, respectively. One EPN species can be hosted by one bacterium species. On the other hand, one bacteria species is symbiotically associated with more than one EPN species (Gulcu, Cimen, Raja R, & Hazir, 2017).

1. Genus *Steinernema*

In 1923, entomopathogenic nematode was first discovered at Germany by Steiner who named it as “*Aplectana kraussei*” (Poinar , & Grewal, 2012; Steiner, 1923). In 1927, Travassos changed its generic name to *Steinernema kraussei*. The entomopathogenic nematodes in genus *Steinernema* are classified in phylum Nematodes, class Chromadorea, order Rhabditida, family Steinernematidae (Travassos, 1927). This nematode is associated with bacteria in genus *Xenorhabdus* that located in the intestinal vesicle of its infective juvenile (Figure 1) (Ciche, Darby, Ehlers, Forst, & Goodrich-Blair, 2006). At present, 108 species of *Steinernema* have been described globally (Table 1).

Figure 1 *Xenorhabdus nematophila* cells situated within the intestinal vesicle (arrow) of *Steinernema carpocapsae* infective juveniles

Source: Ciche et al., 2006

Table 1 List of entomopathogenic nematode in genus *Steinernema*

Genus	Species	References
Steinernema	*abbasi*	(Elawad, Ahmad, & Reid, 1997)
	aciari	(Qiu, Yan, Zhou, Nguyen, & Pang, 2005)
	affine	(Wouts, Mráček, Gerdin, & Bedding, 1982)
	africanum	(Machado et al., 2022)
	akhursti	(Qiu, Hu, Zhou, Mei, et al., 2005)
	anatoliense	(Hazir, Stock, & Keskin, 2003)
	apuliae	(Triggiani, Mrácek, & Reid, 2004)
	arasbaranense	(Nikdel, Niknam, & Wen Ye, 2011)
	arenarium	(Wouts et al., 1982)
	ashiuense	(Phan, Takemoto, & Futai, 2006)
	asiaticum	(Anis, Fayyaz, Reid, & Rowe, 2002)
	australe	(Edgington, Buddie, Tymo, Hunt, et al., 2009)
	backanense	(Phan, Spiridonov, Subbotin, & Moens, 2006)
	balochiense	(Fayyaz et al., 2015)
	batswanae	(Didiza, Lephoto, & Gray, 2021)
	beddingi	(Qiu, Hu, Zhou, Pang, & Nguyen, 2005)
	beitlechemi	(Çimen et al., 2016)
	bertusi	(Katumanyane, Malan, Tiedt, & Hurley, 2020)
	bicornutum	(Tallosi, Peters, & Ehlers, 1995)
	biddulphi	(Cimen et al., 2016)
	bifurcatum	(Fayyaz et al., 2014)
	boemarei	(Lee, Sicard, Skeie, & Stock, 2008)
	borjomiense	(Gorgadze et al., 2018)

Genus	Species	References
Steinernema	*brazilense*	(Nguyen, Ginarte, Leite, Santos, & Harakava, 2010)
	cameroonense	(Kanga et al., 2012)
	carpocapsae	(Wouts et al., 1982)
	caudatum	(Xu, Wang, & Li, 1991)
	ceratophorum	(Jian, Reid, & Hunt, 1997)
	changbaiense	(Ma, Chen, De Clercq, Han, & Moens, 2002)
	cholashanense	(Nguyen, Půža, & Mráček, 2008)
	citrae	(Stokwe, Malan, Nguyen, Knoetze, & Tiedt, 2011)
	colombiense	(López-Núñez, Plichta, Góngora-Botero, & Stock, 2008)
	costaricense	(Uribe-Lorío, Mora, & Stock, 2007)
	cubana	(Mráček, Hernández, & Boëmare, 1994)
	cumgarense	(Phan, Spiridonov, et al., 2006)
	diaprepesi	(Nguyen , & Duncan, 2002)
	eapokense	(Phan, Spiridonov, et al., 2006)
	ethiopiense	(Tamiru et al., 2012)
	everestense	(Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar, & Moens, 2011b)
	fabii	(Abate et al., 2016)
	feltiae	(Wouts et al., 1982)
	glaseri	(Wouts et al., 1982)
	goweni	(San-Blas, Morales-Montero, Portillo, Nermuť, & Puza, 2016)
	guangdongense	(Qiu, Fang, Zhou, Pang, & Nguyen, 2004)

Genus	Species	References
Steinernema	*hebeiense*	(Chen, Li, Yan, Spiridonov, & Moens, 2006)
	hermaphroditum	(Stock, Griffin, & Chaerani, 2004)
	huense	(Phan, Mráček, Půža, Nermut, & Jarošová, 2014)
	ichnusae	(Tarasco, Mráček, Nguyen, & Triggiani, 2008)
	innovationi	(Çimen, Lee, Hatting, Hazir, & Stock, 2014a)
	intermedium	(Poinar, 1985)
	jeffreyense	(Malan, Knoetze, & Tiedt, 2015)
	jollieti	(Spiridonov, Krasomil-Osterfeld, & Moens, 2004)
	kandii	(Godjo et al., 2019)
	karii	(Waturu, Hunt, & Reid, 1997)
	khoisanae	(Nguyen, Malan, & Gozel, 2006)
	khuongi	(Stock, Campos-Herrera, El-Borai, & Duncan, 2018)
	kraussei	(Travassos, 1927)
	kushidai	(Mamiya, 1988)
	lamjungense	(Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar, & Moens, 2011a)
	leizhouense	(Nguyen, Qiu, Zhou, & Pang, 2006)
	litorale	(Yoshida, 2004)
	loci	(Phan, Nguyen, & Moens, 2001a)
	longicaudum	(Shen , & Wang, 1991)
	meghalayensis	(Ganguly, Rathour, Kumar, & Singh, 2011)
	minutum	(Maneesakorn, Grewal, & Chandrapatya, 2010)
	monticolum	(Stock, Choo, & Kaya, 1997)

Genus	Species	References
Steinernema	*neocurtillae*	(Nguyen , & Smart, 1992)
	nepalense	(Khatri-Chhetri, Waeyenberge, Spiridonov, Manandhar, & Moens, 2011c)
	nyetense	(Kanga et al., 2012)
	oregonense	(Liu , & Berry, 1996b)
	populi	(Tian et al., 2022)
	phyllophagae	(Nguyen , & Buss, 2011)
	puertoricense	(Román , & Figueroa, 1994)
	pui	(Qiu, Zhao, Wu, Lv, & Pang, 2011)
	puntauvense	(Uribe-Lorío et al., 2007)
	pwaniensis	(Půža, Nermut, Mráček, Gengler, & Haukeland, 2016)
	rarum	(Doucet, 1986)
	riobrave	(Cabanillas, Poinar, & Raulston, 1994)
	riojaense	(Půža et al., 2020)
	ritteri	(Doucet , & Doucet, 1990)
	robustispiculum	(Phan, Subbotin, Waeyenberge, & Moens, 2005)
	sacchari	(Nthenga, Knoetze, Berry, Tiedt, & Malan, 2014)
	sandneri	(Lis et al., 2021)
	sangi	(Phan, Nguyen, & Moens, 2001b)
	sasonense	(Phan, Spiridonov, et al., 2006)
	scapterisci	(Nguyen , & Smart, 1990)
	scarabaei	(Stock , & Koppenhöfer, 2003)
	schliemanni	(Spiridonov, Waeyenberge, & Moens, 2010)
	serratum	(Liu, 1992)
	siamkayai	(Stock, Somsook, & Reid, 1998)

Genus	Species	References
Steinernema	*sichuanense*	(Mráček, Nguyen, Tailliez, Boemare, & Chen, 2006)
	silvaticum	(Sturhan, Spiridonov, & Mráček, 2005)
	surkhetense	(Khatri-Chhetri et al., 2011c)
	taiwanensis	(Tseng, Hou, & Tang, 2018)
	tami	(Luc, Nguyen, Reid, & Spiridonov, 2000)
	texanum	(Nguyen, Stuart, Andalo, Gozel, & Rogers, 2007)
	thailandensis	(Tangchitsomkid, 1998)
	thanhi	(Phan et al., 2001a)
	thermophilum	(Ganguly , & Singh, 2000)
	tielingense	(Ma, Chen, Li, et al., 2012)
	tophus	(Çimen, Lee, Hatting, Hazir, & Stock, 2014b)
	unicornum	(Edgington, Buddie, Tymo, France, et al., 2009)
	vulcanicum	(Clausi, Longo, Rappazzo, Tarasco, & Vinciguerra, 2011)
	websteri	(Cutler , & Stock, 2003)
	weiseri	(Mrácek, Sturhan, & Reid, 2003)
	xiebinense	(Ma, Chen, De Clercq, et al., 2012)
	xueshanense	(Mráček, Qi-zhi, & Nguyen, 2009)
	yirgalemense	(Nguyen, Tesfamariam, Gozel, Gaugler, & Adams, 2004)

2. Genus *Heterorhabditis*

Heterorhabditis was first reported in 1975 at Australia which was classified in phylum Nematodes, class Chromadorea, order Rhabditida, family Heterorhabditidae (Poinar, 1975). Entomopathogenic nematodes in the genus *Heterorhabditis* were symbiotically associated with bacteria in genus *Photorhabdus*

that live in the anterior and mid intestine of infective juvenile (Figure 2) (Ciche et al., 2006). At present, 30 species of *Heterorhabditis* have been discovered (Table 2).

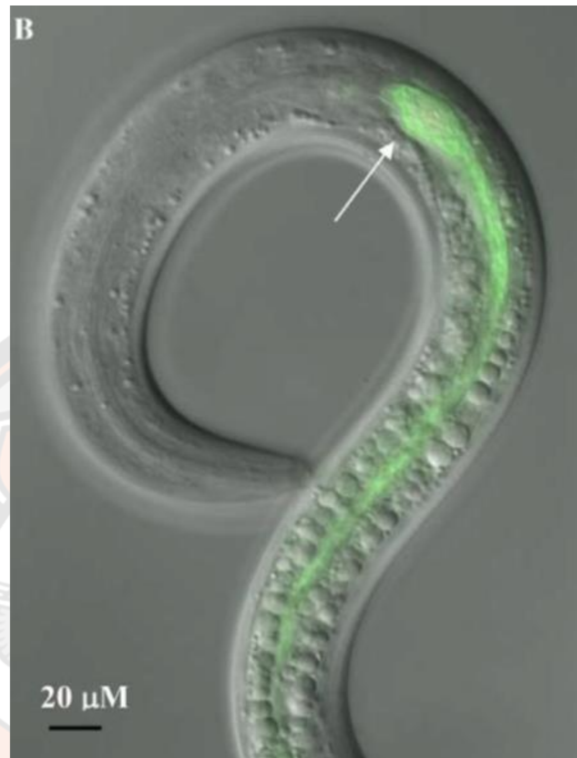

Figure 2 *Photorhabdus luminescens* cells situated within the in the anteriors and midintestine (arrow) of *Heterorhabditis bacteriophora*

Source: Ciche et al., 2006

Table 2 List of entomopathogenic nematode in genus *Heterorhabditis*

Genus	Species	References
Heterorhabditis	*amazonensis*	(Andaló, Nguyen, & Moino, 2006)
	argentinensis	(Stock, 1993)
	atacamensis	(Edgington et al., 2010)
	bacteriophora	(Poinar, 1975)
	baujardi	(Phan, Subbotin, Nguyen, & Moens, 2003)
	beicherriana	(Li, Liu, NERMUŤ, PŮŽA, & MRÁČEK, 2012)
	brevicaudis	(Liu, 1994)
	downesi	(Stock, Griffin, & Burnell, 2002)
	floridensis	(Nguyen, Gozel, Koppenhöfer, & Adams, 2006)
	georgiana	(Plichta, Joyce, Clarke, Waterfield, & Stock, 2009)
	gerrardi	(Plichta et al., 2009)
	hambletoni	(Poinar, 1975)
	hawaiiensis	(Gardner, Stock, & Kaya, 1994)
	heliothidis	(Poinar, 1975)
	hepialius	(Stock, Strong, & Gardner, 1996)
	hoptha	(Poinar, 1979)
	indica	(Poinar, Karunakar, & David, 1992)
	marelatus	(Liu , & Berry, 1996a)
	megidis	(Poinar, Jackson, & Klein, 1987)
	mexicana	(Nguyen, Sharpiro-Ilan, et al., 2004)
	noenieputensis	(Malan, Nguyen, Waal, & Tiedt, 2008)
	pakistanense	(Shahina, Tabassum, Salma, Mehreen, & Knoetze, 2017)
	poinari	(Kakulia , & Mikaia, 1997)

Genus	Species	References
Heterorhabditis	*ruandica*	(Machado, Bhat, et al., 2021)
	safricana	(Malan et al., 2008)
	somsookae	(Maneesakorn, An, Grewal, & Chandrapatya, 2015)
	sonorensis	(Stock, Rivera-Orduño, & Flores-lara, 2009)
	taysearae	(Shamseldean, Abou-El-Sooud, Abd-Elgawad, & Saleh, 1996)
	zacatecana	(Machado, Bhat, et al., 2021)
	zealandica	(Poinar, 1990)

3. Life cycle of entomopathogenic nematodes (EPNs)

The infective juvenile (IJ), a free living stage of EPNs, lives in the soil. *Steinernema* penetrates insect larva via natural opening such as mouth, anus and spiracle while *Heterorhabditis* pass through by grating the intersegmental membranes using a dorsal tooth. The IJ releases symbiotic bacteria cells into the haemocoel of insects. The bacteria multiply, release toxins and exoenzymes. This cause the insect larva die within 24-48 h. The IJ grows and develops to the 4th stage larvae and adult within 2-3 days. Reproduction of nematodes continue for 2-3 generations, until the nutrient in insect cadaver deplete. The development from IJ to adult is inhibited then, the IJ accumulates. At last, the IJ (non-feeding stage) leave insect cadaver into the soil to find the new insect hosts. These IJ survive for 2-3 months without the host (Figure3). For *Steinernema*, reproduction is amphimictic which is the union of male and female gamete. The IJ of *Steinernema* develops to either male or female adult. In contrast, the IJ of *Heterorhabditis* develop to first generation hermaphrodite female and this female generate second generation of amphimictic male and female. *Steinernema carpocapsae* in the first- and second-generations lay a larger number of their eggs than *H. bacteriophora*. The eggs of *S. carpocapsae* from the third-generation females develope via endotokia matricida. The IJ generated from

endotokia matricida of *S. carpocapsae* do not develop into the IJ up to they had exited from the body of the mother nematode (Burnell, & Stock, 2000) (Figure 3). Kakulia, G., & Mikaia, N. (1997) New species of the nematode Het-erorhabditis poinari sp. nov. (Rhabditida, Heterorhabditidae). Bulletin of the Georgian Academy of Sciences, 155, 457-459

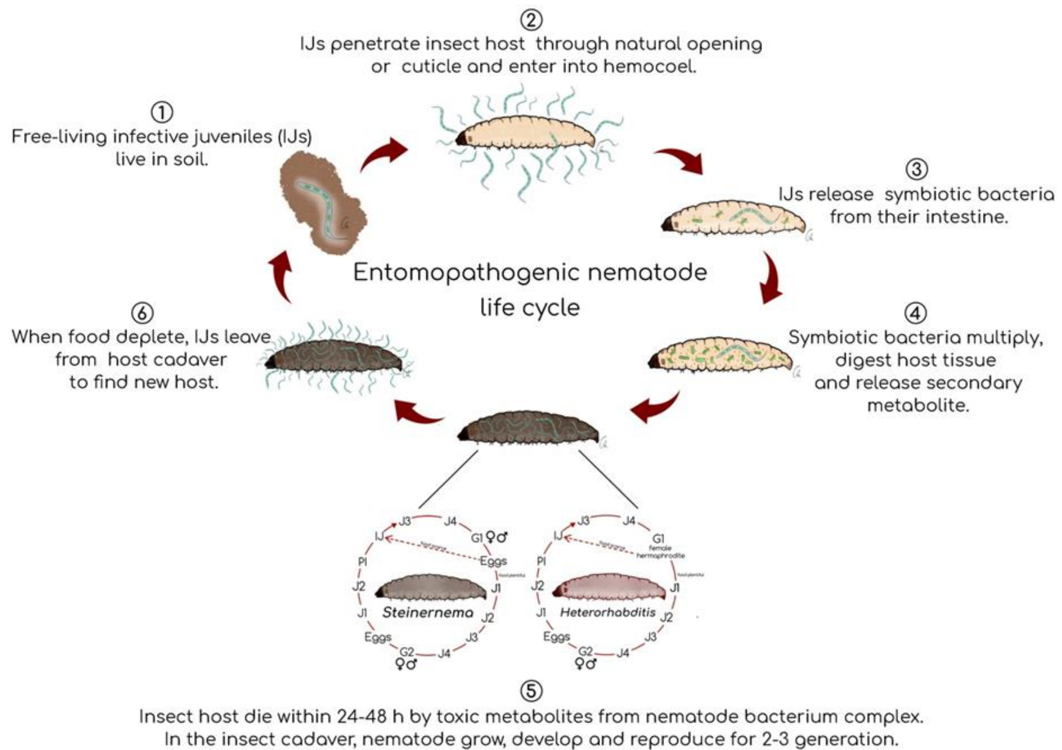

Figure 3 The life cycle of entomopathogenic nematodes

4. Identification of entomopathogenic nematodes (EPNs)

Morphological identification of EPNs could be performed by using scanning electron microscope (SEM) for distinguishing external structure of infective stage, males and females.

Morphology of *Steinernema* when observed by SEM shows in Figure 4, 5 and 6. Males have smaller size than females. They have one testis and one pair of spicules with long gubernaculum but without bursa. Tail tip has rounded, digitate or mucronate shape. Moreover, a single and 10 to 14 pairs of genital papillae present with 7 to 10 pairs precloacal. Meanwhile, females have large size and smooth or annulated cuticle. Lateral fields are absent. Head rounded or ringed with six lips. The female reproductive system has 2 ovaries (didelphic) which located at anterior and posterior part of body (amphidelphic). Vulva located at mid-body, with or without epiptygma. Tail is longer or shorter than anal body width. The IJ has slender shape with or without a sheath. Esophagus and intestine seem to narrow tube. Digestive system is not active. Tail has conoid or filiform shape. Phasmids locate at mid-tail. Moreover, group of *Steinernema* can distinguish base on morphology which showed in Table 3 (Nguyen, 2010b).

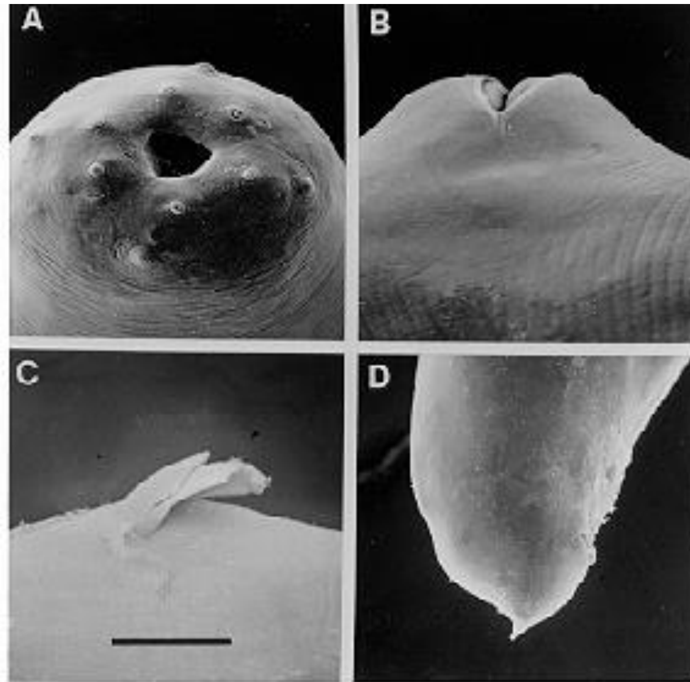

Figure 4 Structures of *Steinernema* female A) Face view, B) Vulva, C) Epiptygma and D) Female tail

Source: Nguyen, 2010b

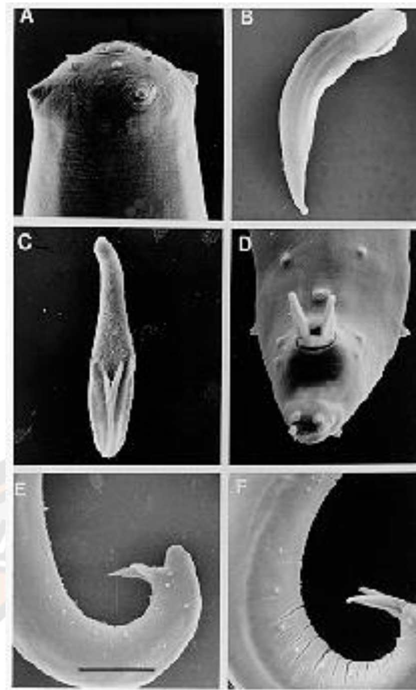

Figure 5 Structures of *Steinernema* males

Source: Nguyen, 2010b

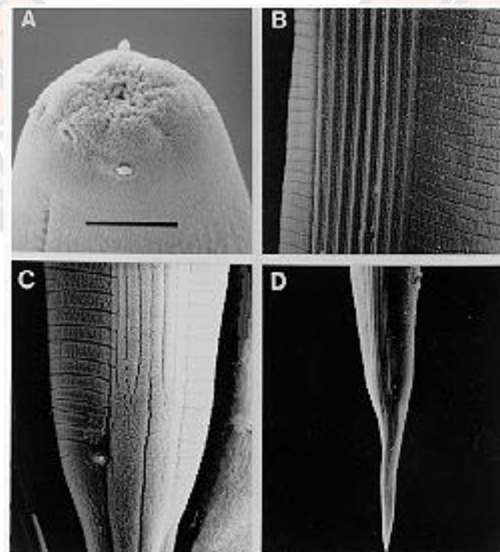

Figure 6 Structures of infective juvenile of *Steinernema glaseri*

Source: Nguyen, 2010b

Table 3 Classification of *Steinernema* group based on morphology of IJ stage

Group	Morphology	*Steinernema* species
glaseri	Length of IJ more than 1,000 µm	*Steinernema aciari*
		Steinernema apuliae
		Steinernema arenarium
		Steinernema australe
		Steinernema boemarei
		Steinernema brazilense
		Steinernema caudatum
		Steinernema cubana
		Steinernema diaprepesi
		Steinernema ethiopiense
		Steinernema glaseri
		Steinernema guangdongense
		Steinernema hermaphroditum
		Steinernema innovation
		Steinernema khoisanae
		Steinernema khuongi
		Steinernema lamjungense
		Steinernema leizhouense
		Steinernema longicaudum
		Steinernema phyllophagae
		Steinernema puertoricense
		Steinernema pui
		Steinernema tophus
		Steinernema vulcanicum

Group	Morphology	*Steinernema* species
feltiae	Length of IJ between 700 to 1,000 µm	*Steinernema akhursti*
		Steinernema ashiuense
		Steinernema beitlechemi
		Steinernema jeffreyense
		Steinernema changbaiense
		Steinernema cholashanense
		Steinernema citrae
		Steinernema ethiopense
		Steinernema everestense
		Steinernema fabii
		Steinernema feltiae
		Steinernema hebeiense
		Steinernema ichnusae
		Steinernema jollieti
		Steinernema karii
		Steinernema kraussei
		Steinernema litorale
		Steinernema loci
		Steinernema monticolum
		Steinernema neocurtillae
		Steinernema oregonense
		Steinernema puntauvense
		Steinernema robustispiculum
		Steinernema sacchari
		Steinernema sangi
		Steinernema scarabaei
		Steinernema schliemanni
		Steinernema silvaticum
		Steinernema texanum
		Steinernema thanhi

Group	Morphology	*Steinernema* species
feltiae	Length of IJ between 700 to 1,000 µm	*Steinernema tielingense*
		Steinernema unicornum
		Steinernema weiseri
		Steinernema xinbinense
		Steinernema xueshanense
intermedium	Length of IJ between 600 to 700 µm	*Steinernema affine*
		Steinernema anatoliense
		Steinernema arasbaranense
		Steinernema asiaticum
		Steinernema backanense
		Steinernema balochiense
		Steinernema beddingi
		Steinernema cameroonense
carpocapsae	Length of IJ less than 600 µm	*Steinernema carpocapsae*
		Steinernema colombiense
		Steinernema cumgarense
		Steinernema eapokense
		Steinernema huense
		Steinernema intermedium
		Steinernema kushidai
		Steinernema meghaleyense
		Steinernema minutum
		Steinernema nepalense
		Steinernema nyetense
		Steinernema pwaniensis
		Steinernema rarum
		Steinernema ritteri
		Steinernema sasonense
		Steinernema scapterisci

Group	Morphology	*Steinernema* species
carpocapsae	Length of IJ less than 600 µm	*Steinernema siamkayai* *Steinernema sichuanense* *Steinernema surkhentense* *Steinernema tami* *Steinernema thermophilum* *Steinernema websteri*
bicornutum	IJ has 2 horn-like structures	*Steinernema abbasi* *Steinernema bicornutum* *Steinernema biddulphi* *Steinernema bifurcatum* *Steinernema ceratophorum* *Steinernema costaricense* *Steinernema goweni* *Steinernema pakistanense* *Steinernema riobrave* *Steinernema yirgalomense*

On the other side, female of *Heterorhabditis* spp. divided into 2 types, hermaphroditic and amphimictic females. Hermaphroditic female has head truncated to slightly rounded head with six conical lips. Stoma has wide and shallow appearance. Vulva has slit-like shape which locate at slight anterior to mid-body. Tail has pointed shape, longer than anal body width. Amphimictic female is similar to hermaphroditic female, but usually smaller than it. Vulva is function for mating only. Male has one testis. Spicules are paired and slightly curved shape. Gubernaculum long as spicule length. Bursa are peloderan appearance with nine pairs of genital papillae. IJ usually has sheath with anterior tessellate pattern and longitudinal ridges. Mouth and anus closed. Esophagus and intestine similar to narrow tube. Excretory pore located at posterior before nerve ring. Tail is pointed shape. Morphology of *Heterorhabditis* spp. when observed by SEM showed in Figure 7 (Nguyen, 2010a).

However, similar morphology in several EPN species is the limitation to distinguish into species level. Moreover, SEM are expensive and require special training to operate. The preparation of sample has many steps and be difficult. Therefore, alternative techniques have been developed for using in the identification of EPNs. Molecular techniques have been used to classify the species of EPNs in genera *Steinernema* and *Heterorhabditis*. There were several molecular methods using for identificatioin of EPNs. The restriction enzymes for restricting 26 rDNA gene and internal transcribed spacer (ITS) were used in restriction fragment length polymorphism (RFLP). This technique can classify EPN in species level but it requires a lot of enzymes (Nasmith, Speranzini, Jeng, & Hubbes, 1996). In addition, DNA sequencing of 18S rDNA was used to identification of EPNs. This method can distinguish *Steinernema* and *Heterorhabditis*, but inability to distinguish between species level cause of low variation (Blaxter et al., 1998). In addition, sequencing of nucleotide fragments was used to identify EPNs. The 28s rDNA sequence can differentiate EPNs in species level of genus *Steinernema* (Nguyen, 2010b; Stock, Campbell, & Nadler, 2001; Thanwisai et al., 2012). The nucleotide sequence at ITS rDNA whic is high variation, thus DNA sequencing of this fragment can differentiate EPNs in species level of genus *Heterorhabditis* (Hominick et al., 2009; Thanwisai et al., 2012). Moreover, phylogenetic study based on nucleotide sequences of specific fragments can be analyzed evolutionary relationships (Adams, Burnell, & Powers, 1998; Nguyen, Sharpiro-Ilan, et al., 2004; Nguyen, Maruniak, & Adams, 2001; Stock et al., 2001; Thanwisai et al., 2012).

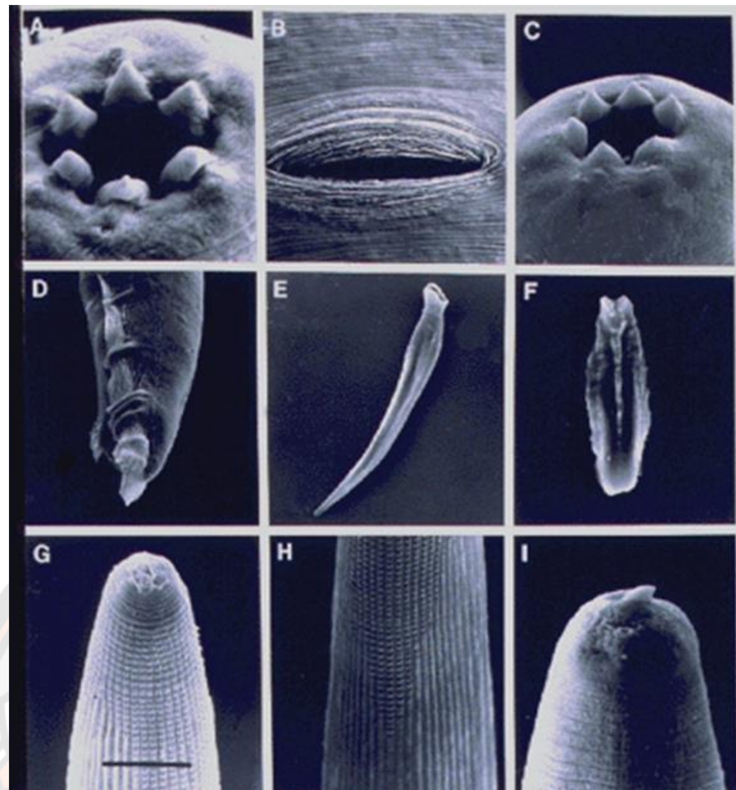

Figure 7 Structures of *Heterorhabditis* spp. A) Hermaphroditic female face view, B) Vulva of hermaphroditic female, C) Amphimictic female face view, D) Male tail with peloderan bursa, E) Spicule, F) Gubernaculum, G-I) Anterior region of IJ

Source: Nguyen, 2010a

5. Phylogenetic relationship of entomopathogenic nematodes (EPNs)

For genetic relationship analysis of EPNs, internal transcribed spacer (ITS) rDNA and 28S rRNA have been used for study genetic relationship of *Heterorhabditis* and *Steinernema*, respectively. (Fukruksa, 2014; Hominick et al., 2009; Nguyen, 2010b; Stock et al., 2001; Suwannaroj, 2014; Thanwisai et al., 2012; Yimthin, 2014). In 2012, Thanwisai studied diversity and analysis of genetic relationships by maximum-likelihood method of EPNs collected from 13 provinces of Thailand. Most of *Steinernema* isolates in Thailand were closely related to *S. websteri* strain AS1 and the minor *Steinernema* isolates were closely related to *S. khoisanae*. *Heterorhabditis* Thai isolates were closely related to *H. indica* strain 95, *H. indica* strainN-MP111, *H. indica* isolate CICR- COTNG2, and *H. indica* isolate Khatatba and some were closely related to *H. baujardi*, *H. bacteriophora* isolate 117-C, *Heterorhabditis* sp. SGgj, and *Heterorhabditis* sp. SGmg3 (Thanwisai et al., 2012). The genetic relationships of EPNs in upper northern Thailand were also found that *Steinernema* isolates were closely related with *S. websteri* strain AS1 and *S. scarabaei*. In addition, *Heterorhabditis* isolates in the upper northern Thailand were closely related with *H. indica* isolate CICR-BBFN Warud, *Heterorhabditis* sp. PAK.SH.123, and *Heterorhabditis* sp. SGmg3 (Fukruksa, 2014). Moreover, *Steinernema* isolates in the northeast Thailand were closely related to *S. websteri*, *S. abbasi*, and *S. sangi* while *Heterorhabditis* isolates were closely related to *H. indica* isolate CICR-BBFN Warud, *H. baujardi*, and *Heterorhabditis* sp. SGmg3 (Yimthin, 2014). The genetic relationships of EPNs in Thailand including Phitsanulok, Chaiyaphum, Nakhon Ratchasima, and Saraburi Provinces were also noted with Steinernema isolates closely related to *S. websteri* strain AS1. In addition, *Heterorhabditis* isolates from these provinces were closely related with *H. indica* isolate CICR-BBFN Warud and *Heterorhabditis* sp. SGmg3 (Suwannaroj, 2014). *Steinernema* isolates from Nakhon Sawan and Uthai Thani Provinces were closely related to *S. websteri* JCI032 (Vitta et al., 2015). Furthermore, entomopathogenic nematode isolates in upper north of Thailand were closely related to *S. websteri* JCI032, *S. scarabaei*, *H. indica*, *H. gerrardi*, and *Heterorhabditis* sp. SGmg3 (Vitta et al., 2017). In addition, the genetic relationship of EPNs collected from Mae Wong National Park were closely related to *S. websteri*, *S. kushidai*, *H. indica*, *H. baujardi*,

and *H. zealandica* (Muangpat et al., 2017). The EPNs found within Nam Nao National Park were exhibited a close genetic relationship to *S. websteri* and *H. baujardi* (Yooyangket et al., 2018)

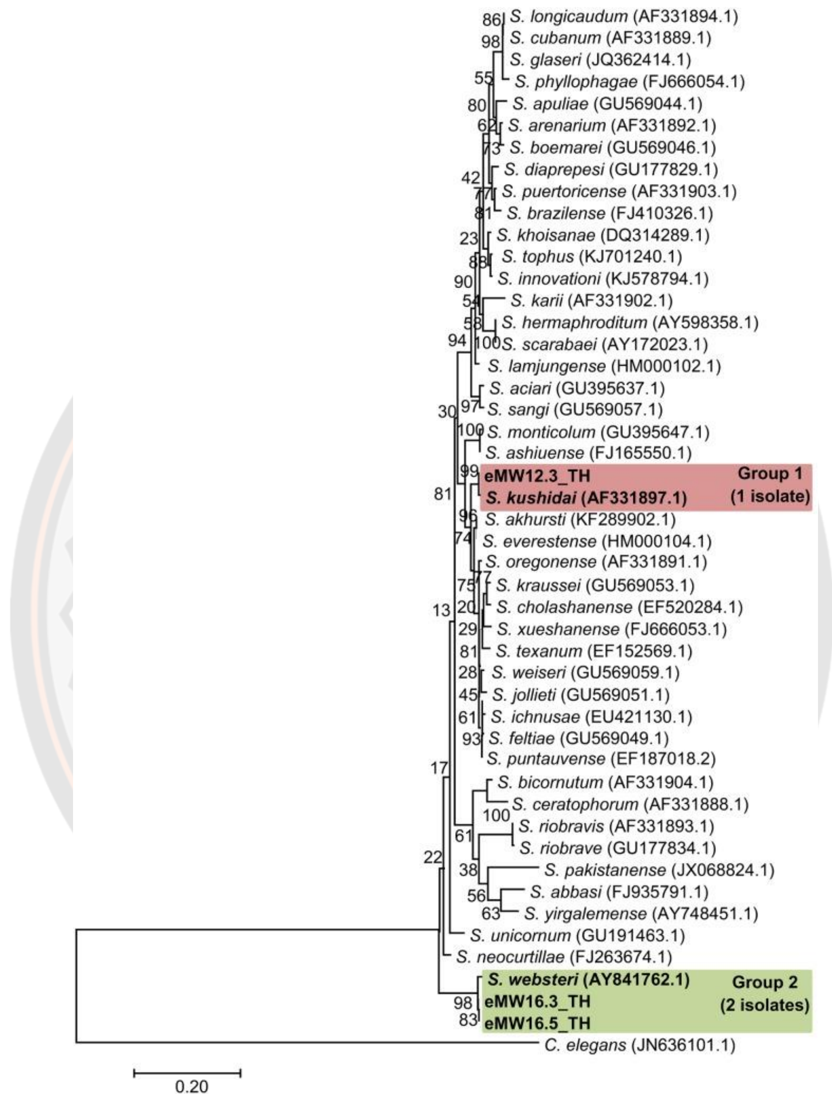

Figure 8 Maximum likelihood tree for *Steinernema*. The phylogenetic tree was constructed based on a partial region of the 28S rRNA gene (634 bp) for *Steinernema* isolates from Mae Wong National Park, Kamphaeng Phet Province, Thailand in conjunction with 44 sequences of *Steinernema* 28S rRNA gene downloaded from NCBI. *Caenorhabditis elegans* was used as the out-group

Source: Muangpat et al., 2017

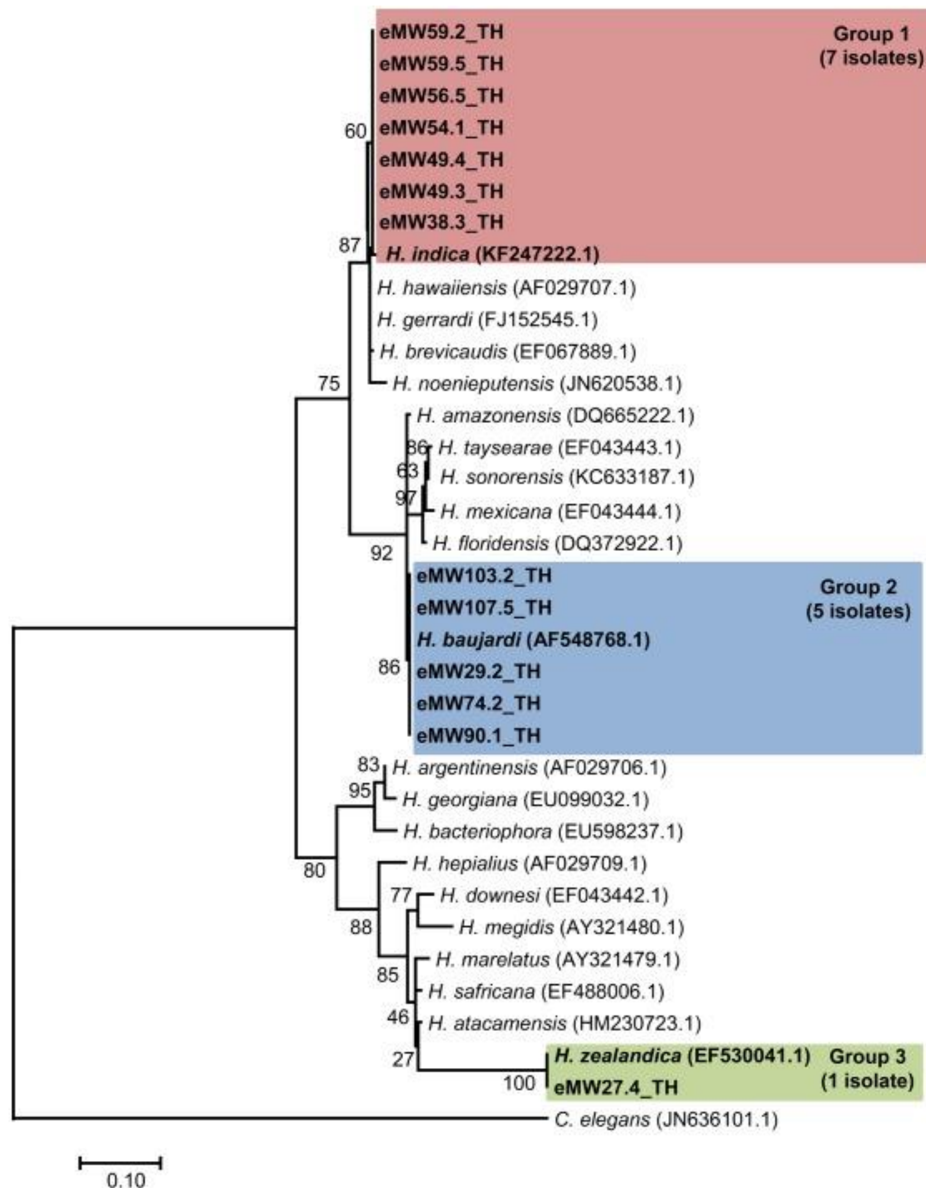

Figure 9 Maximum likelihood tree for *Heterorhabditis*. The phylogenetic tree was constructed based on a partial region (634 bp) of the internal transcribed spacer (ITS) for 13 *Heterorhabditis* isolates from Mae Wong National Park, Kamphaeng Phet Province, Thailand in conjunction with 20 sequences of the *Heterorhabditis* ITS regions downloaded from NCBI. *C. elegans* was used as the out-group

Source: Muangpat et al., 2017

6. Diversity and factor of distribution and survival

Entomopathogenic nematodes are global distribution with different species according to geographic regions (de Brida et al., 2017). In natural habitats of California, USA. *Steinernema carpocapsae*, *S. feltiae*, *S. kraussei*, *S. longicaudum*, *S. oregonense*, *H. marelatus*, and *H. bacteriophora* were identified in this area. *Steinernema kraussei* and *S. feltiae* were the predominant findings within acidic soils in organic matter (Stock, Pryor, & Kaya, 1999). In 2009, 1500 soil samples obtained from diverse habitats situated across seven distinct geographic regions within South Africa were evaluated for the surveying of EPN. *Steinernema khoisanae* and *H. bacteriophora* were recovered (Hatting, Patricia Stock, & Hazir, 2009). Tarasco and collaborator investigated EPN diversity in Italy, who found 12 species of EPNs including *H. bacteriophora*, *H. downesi*, *H. megidis*, *Steinernema feltiae*, *S. affine*, *S. kraussei, S. apuliae*, *S. ichnusae*, *S. carpocapsae*, *S. vulcanicum*, *Steinernema* 'isolate S.sp.MY7' of *S. intermedium* group, and *S. arenarium*. *Steinernema feltiae* and *H. bacteriophora* are the most commonly encountered species (Tarasco et al., 2014). In 2017, diversity of EPNs in agricultural areas of Brazil were discovered with *H. amazonensis* and *S. rarum* found (de Brida et al., 2017).

In Thailand, EPNs are widely distribution throughout the country. The common species of EPNs found are *S. websteri* and *H. indica*. Almost EPNs were found in loam at 26ºC - 33ºC and pH values of 5.0-7.0. *Steinernema websteri*, *S. scarabaei*, *H. indica*, *H. gerrardi* and *Heterorhabditis* sp. SGmg3 were also found in the upper northern Thailand. Entomopathogenic nematodes were isolated from loam at 24°C -38°C, pH values of 1.5-7.0 and a soil moisture content of 0.5-6.8% (Vitta et al., 2017; Vitta et al., 2015). In 2017, Muangpat reported that *H. indica*, *H. baujardi*, *H. zealandica, S. websteri* and *S. kushidai* were discovered in Mae Wong Nation Park, Kamphaeng Phet Province, Thailand (Muangpat et al., 2017). Moreover, soil sample in Nam Nao National Park, Phetchabun province, Thailand were isolated for EPNs surveying. *H. baujardi* and *S. websteri* were found in this area. The majority of EPNs were extracted from loam soil at 19°C - 30°C, with the soil pH values of 4.8-7.0 and soil moisture ranging between 1.0 and 8.0 (Yooyangket et al., 2018). Recenlty, report in 2020 revealed that *H. indica*, *Heterorhabditis* sp. SGmg3 and

S. surkhetense were discovered in nine districts of Phitsanulok Province, Thailand (Suwannaroj et al., 2020)

The infective juveniles of EPNs live in the soil for a long time even in the lack of food condition. The survival of EPNs depend on the intrinsic factors, i.e., behavior, physical characteristic, and genetic characteristic, and extrinsic factors, i.e., temperature, humidity, natural enemies, sunlight, and soil texture (Kaya, 1990). Soil characteristics are one of the important factors affecting survival of EPNs. The soil texture that suitable for EPNs is sandy loam (Raheel, Javed, Khan, & Ahmad, 2016). In this soil, EPNs can move better than clay because it has fewer gaps between soil and less oxygen (Kung, Gaugler, & Kaya, 1991). Soil humidity is another important factor. EPNs require water for movement. The appropriate humidity is between 8 and 25% (Grant , & Villani, 2003). In addition, temperature is important factor affecting growth, reproduction and survival. The optimal temperature range is 25-28 ºC (Kaya, 1997). Acidity is one of the factors affecting the survival of EPNs. The pH of the soil ranging between 4 and 8 does not affect to EPNs but pH is greater than 10 it can be harmful to EPNs (Kaya, 1990). In addition, UV light inhibited the reproduction and development of *S. carpocapsae* after exposure for 7 min (Gaugler, & Boush, 1978).

7. Application for control insects

EPNs in genus *Steinernema* and *Heterorhabditis* are insect parasite causing insect death with toxic metabolites that released by nematode bacterium complex. Pathogenicity of EPNs to insect pest is different due to several factors including species of EPNs and their symbiotic bacteria. Also, it depends on the age of insect pest. Previous study in 1992, *H. indica* have potential to kill insect pest of sugarcans, *Holotricha serrate* and *Leucopholis lepidophora* (Poinar et al., 1992). Two year later, EPNs were used for control army worm, stem borrers, web worms, root weevil, and flea beetles (Georgis, & Manweiler, 1994). In 2006, *S. scarabaei*, *H. bacteriophora* (strain GPS11), *H. bacteriophora* (strain TF) and *H. zealandica* (strain X1) were reported as the bio-agent potential to control Japanese beetle (*Popillia japonica*) (Koppenhöfer, Grewal, & Fuzy, 2006). In 2018, *H. bacteriophora* and *S. carpocapsae* have ability to manage the tomato leaf miner (*Tuta absoluta*) when treated in greenhouse (Kamali, Karimi, & Koppenhöfer, 2018). In addition, *Steinernema* sp. 64-2, four isolates of *S. carpocapsae* (isolate A24, All, Mex, and G-

R3a-s), *S. longicaudum* (Shen , & Wang, 1991) X-7, and two isolates of *H. indica* (212-2 and LN2) have potential effective against the instar larvae; the second, third, and fourth of the tobacco cutworm (*Spodoptera litura*) (Yan et al., 2019).

Moreover, several researches were reported on the pathogenic potential of EPNs against medical important insects. In the laboratory setting, *H. bacteriophora* HP88 and *H. baujardi* LPP7 demonstrated effectiveness against the juvenile stage of the stable fly *(Stomoxys calcitrans)*. Larval mortality exhibited significance ($p<0.05$) across all EPN concentrations; 25, 50, 100, 150, and 200 EPNs/larvafor both EPN strains when compared to the control groups. The concentrations of *H. bacteriophora* HP88 resulted in an LC50 of 0.36 EPN/larva and an LC90 of 29.1 EPN/larva, whereas H. baujardi LPP7 yielded an LC50 of 39.85 EPN/larva and an LC90 of 239.18 EPN/larva (Leal, Monteiro, Mendonça, Bittencourt, & Bittencourt, 2017a). Experiments assessing the efficacy of EPNs against *Ae. aegypti*, *Anopheles stephensi*, and *Culex quinquefasciatus* demonstrated that *S. abassi* exhibited the highest mortality rate against *Ae. aegypti* (97.33%). For *A. stephensi*, *Heterorhabditis indica* (KPR-8) displayed a mortality rate of 97.33%, while *S. siamkayai* (KPR-4) yielded a mortality rate of 98.67% against *C. quinquefasciatus*. (Dilipkumar et al., 2019). The pathogenicity of EPNs against housefly (*Musca domestica*) was evaluated. Adult mortality rate were significantly differences ($p=0.0001$) among nematode species. In addition, *H. indica* induced the highest mortality (53.3%) when tested on peat moss (Arriaga , & Cortez-Madrigal, 2018).

Symbiotic bacteria

1. *Xenorhabdus*

Xenorhabdus was first reported by George and Thomas in 1965 within intestine of EPN in family Steinernematidae. At that time, the bacteria were classified in genus *Achromobacter* and in family Enterobacteriaceae. Then, it was changed to genus *Xenorhabdus* (George, & Thomas, 1979). *Xenorhabdus* is Gram negative bacilli bacteria, facultative anaerobic and motile with peritrichous flagella. The formation of phenotypic variants forms includes the primary and secondary form that differ in biochemical properties (Akhurst , & Boemare, 1988). The primary form of *Xenorhabdus* produces antibiotics such as hydroxystilbense polyketides for inhibiting

other organisms that digest host insect tissue (Boemare, Akhurst, & Mourant, 1993). Moreover, primary form absorbs certain dyes, and develops larger intracellular inclusion (crystalline protein) than secondary form (Forst et al., 1997). Secondary form produces metabolites that stimulate EPNs for sexual reproduction. The colony characteristics on nutrient- bromothymol blue- triphenyl tetazolium chioride agar (NBTA) containing bromothymol blue and triphenyl tetazolium chioride (TTC) at 25 - 28°C are convex, circular, undulated, blue or brown color from bromothymol blue absorption (Thanwisai et al., 2012). At present, 27 species of *Xenorhabdus* were described (Table 4).

Table 4 List of symbiotic bacteria in genus *Xenorhabdus*

Genus	Species	References
Xenorhabdus	*beddingii*	(Akhurst , & Boemare, 1988)
	bovienii	(Akhurst , & Boemare, 1988)
	budapestensis	Lengyel et al., 2005
	cabanillasii	(Tailliez, Pagès, Ginibre, & Boemare, 2006)
	doucetiae	(Tailliez et al., 2006)
	eapokensis	(Kämpfer, Tobias, Ke, Bode, & Glaeser, 2017)
	ehlersii	(Lengyel et al., 2005)
	griffiniae	(Tailliez et al., 2006)
	hominickii	(Tailliez et al., 2006)
	indica	(Somvanshi et al., 2006)
	innexi	(Lengyel et al., 2005)
	ishibashii	(Kuwata et al., 2013)
	japonica	(Nishimura, Hagiwara, Suzuki, & Yamanaka, 1994)
	khoisanae	Ferreira et al., 2013a
	koppenhoeferi	(Tailliez et al., 2006)

Genus	Species	References
Xenorhabdus	*kozodoii*	(Tailliez et al., 2006)
	lircayensis	(Castaneda-Alvarez, Prodan, Zamorano, San-Blas, & Aballay, 2021)
	magdalenensis	(Tailliez et al., 2006)
	mauleonii	(Tailliez et al., 2006)
	miraniensis	(Tailliez et al., 2006)
	nematophila	(George , & Thomas, 1979)
	poinarii	(Akhurst , & Boemare, 1988)
	romanii	(Tailliez et al., 2006)
	stockiae	(Tailliez et al., 2006)
	szentirmaii	(Lengyel et al., 2005)
	thuongxuanensis	(Kämpfer et al., 2017)
	vietnamensis	(Tailliez et al., 2006)

2. *Photorhabdus*

Photorhabdus is Gram negative bacilli bacteria, facultative anaerobic, motile with peritrichous flagella and is classified in family Enterobacteriaceae. This bacterium has catalase and oxidase activity. *Photorhabdus* have primary and secondary forms. It produces bioluminescence in secondary form (Leisman, Waukau, & Forst, 1995). Colony characteristics on NBTA are convex, circular, entire, green color (Thanwisai et al., 2012). *Photorhabdus* is associated with EPN in family Heterorhabditidae (Forst et al., 1997). At first, *Photorhabdus* was isolated from *H. bacteriophora* and named as *Xenorhabdus luminescens* (Thomas , & Poinar, 1979) but it has catalase activity and production of bioluminescence. Therefore, it was changed into new genus, and named as *Photorhabdus luminescens* (Boemare et al., 1993). Later, two new species of *Photorhabdus* were found, *P. temperata* and *P. asymbiotica* (Fischer-Le Saux, Viallard, Brunel, Normand, & Boemare, 1999). *P. asymbiotica* is the only species of *Photorhabdus* known to be a human pathogen (Wilkinson et al., 2009). According old classification, 4 species with 19 subspecies of *Photorhabdus* were reported worldwide at the present. However, Machado and

collaborators reconstructed the phylogenetic relationships between all described *Photorhabdus* species and subspecies with whole-genome sequencing and traditional techniques and proposed to be renamed (Machado et al., 2018). List of *Photorhabdus* species up to date in old and updated classification are showed in Table 5.

Table 5 List of symbiotic bacteria in genus *Photorhabdus*

Species (Updated classification)	References	Species (Old classification)	References
P. aegyptia	(Machado, Muller, et al., 2021)	-	-
P. akhurstii subsp. *akhurstii*	(Machado, Somvanshi, Muller, Kushwah, & Bhat, 2021)	*P. luminescens* subsp. *akhurstii*	(Fischer-Le Saux et al., 1999)
P. akhurstii subsp. *bharatensis*	(Machado, Somvanshi, et al., 2021)	-	-
P. asymbiotica	(Machado et al., 2018)	*P. asymbiotica* subsp. *asymbiotica*	(Fischer-Le Saux et al., 1999)
P. australis subsp. *australis*	(Machado, Muller, et al., 2021)	*P. asymbiotica* subsp. *australis*	(Akhurst et al., 2004)
P. australis subsp. *thailandensis*	(Machado, Muller, et al., 2021)	-	-
P. caribbeanensis	(Machado et al., 2018)	*P. luminescens* subsp. *caribbeanensis*	(Tailliez et al., 2010)

Species (Updated classification)	References	Species (Old classification)	References
P. cinerea	(Machado et al., 2018)	*P. temperata* subsp. *cinerea*	(Tóth , & Lakatos, 2008)
P. hainanensis	(Machado et al., 2018)	*P. luminescens* subsp. *hainanensis*	(Tailliez et al., 2010)
P. heterorhabditis subsp. *aluminescens*	(Machado, Muller, et al., 2021)	-	-
P. heterorhabditis subsp. *heterorhabditis*	(Machado, Muller, et al., 2021)	*P. heterorhabditis*	(Ferreira et al., 2014)
P. hindustanensis	(Machado, Somvanshi, et al., 2021)	-	-
P. kayaii	(Machado et al., 2018)	*P. luminescens* subsp. *kayaii*	(Hazir et al., 2004)
P. khanii subsp. *khanii*	(Machado et al., 2019)	*P. temperata* subsp. *khanii*	(Tailliez et al., 2010)
P. khanii subsp. *guanajuatensis*	(Machado et al., 2019)	-	-

Species (Updated classification)	References	Species (Old classification)	References
P. kleinii	(Machado et al., 2018)	*P. luminescens* subsp. *kleinii*	(An , & Grewal, 2011)
P. laumondii subsp. *clarkei*	(Machado et al., 2018)	-	-
P. laumondii subsp. *laumondii*	(Machado et al., 2018)	*P. luminescens* subsp. *laumondii*	(Fischer-Le Saux et al., 1999)
P. luminescens subsp. *luminescens*	(Machado et al., 2019)	*P. luminescens* subsp. *luminescens*	(Thomas , & Poinar, 1979)
P. luminescens subsp. *mexicana*	(Machado et al., 2019)	-	-
P. luminescens subsp. *sonorensis*	(Orozco, Hill, & Stock, 2013)	*P. luminescens* subsp. *sonorensis*	(Orozco et al., 2013)
P. namnaonensis	(Machado et al., 2018)	*P. luminescens* subsp. *namnaonensis*	(Glaeser et al., 2016)
P. noenieputensis	(Machado et al., 2018)	*P. luminescens* subsp. *noenieputensis*	(Ferreira et al., 2013)

Species (Updated classification)	References	Species (Old classification)	References
P. stackebrandtii	(Machado et al., 2018)	*P. temperata* subsp. *stackebrandtii*	(An , & Grewal, 2010)
P. tasmaniensis	(Machado et al., 2018)	*P. temperata* subsp. *tasmaniensis*	(Tailliez et al., 2010)
P. temperata	(Machado et al., 2018)	*P. temperata* subsp. *temperata*	(Fischer-Le Saux et al., 1999)
P. thracensis	(Machado et al., 2018)	*P. temperata* subsp. *thracensis*	(Hazir et al., 2004)

3. Life cycle of *Photorhabdus* and *Xenorhabdus*

Photorhabdus and *Xenorhabdus* are similar in the life cycles. *Photorhabdus* live in the anterior and mid intestine of EPN while *Xenorhabdsu* live in the intestinal vesicle of EPN. Bacteria enter to heamolymph of insect larva by EPN infective stage (Adams et al., 2006; Wang, & Gaugler, 1998). The symbiotic bacteria were released into intestine. *Photorhabdus* were released through mouth part while *Xenorhabdus* were released via defecation (Martens, Heungens, & Goodrich-Blair, 2003). Then, symbiotic bacteria multiply, release toxin and exoenzyme that causing septicemia of insect host. Then, insect host die within 48 h (Forst, & Clarke, 2002). *Photorhabdus* rapidly multiply in haemolymph than destroy midgut epithelium of intestine and immune system by release metalloproteinase (Silva et al., 2002). In addition, *Xenorhabdus* release endotoxin from outer membrane such as lipopolysaccharide (LPS) for damage hemocyte of insect host (Brillard, Ribeiro, Boemare, Brehélin, & Givaudan, 2001; Dunphy, & Thurston, 1990). Both bacteria release secondary metabolites that can kill bacteria, fungi, and yeast (Akhurst, 1982). Thus, the death insect hosts having the special characters are late decompose and black or red color (Emelianoff et al., 2008).

4. Identification

Photorhabdus and Xenorhabdus can be identified by various methods. These bacteria are Gram negative bacilli, motile, facultative anaerobic, nonsporulating. Molecular characterization of the 16S rDNA showed that *Xenorhabdus* was closely related to *Phothorhabdus* and they were the sister group of the members of the family Enterobacterioceae (Forst et al., 1997).

4.1 Biochemical test

Biochemical tests use to distinguish *Xenorhabdus* and *Photorhabdus* from other Enterobacteriaceae but inability to distinguish *Xenorhabdus* from *Photorhabdus* due to give negative results for many biochemical tests. However, only in *Photorhabdus* has catalase production, therefore, it gives positive result for catalase activity. Moreover, *Photorhabdus* bacteria produce biolumineacence but *Xenorhabdus* lack of this ability (Akhurst, & Boemare, 1988).

4.2 Molecular techniques

4.2.1 DNA-DNA hybridization

DNA-DNA hybridization is used to compare DNA from two different species and to determine the similarity of DNA from different sources.

This technique used to differentiate between *X. nematophilus* and *X. luminescens* (Grimont et al., 1984). Using nucleic acid probes directed against 16S rDNA, *X. nematophila*, *X. bovienii*, *X. poinarii*, *X. beddingii*, and *X. luminescens* were identified and were distinguished them from other Enterobacteriaceae (Pütz, Meinert, Wyss, Ehlers, & Stackebrandt, 1990).

4.2.2 Ribotyping

RFLP of 16S rDRNA was developed for identification. *Xenorhabdus* spp. required at least 4 endonucleases including *Cfol*, *Hinfl*, *Mspl*, and *Alul* or *HaeIII* or *Ddel* to generate all ribotypes. In the same way, *P. luminescens* required a minimum set of three restriction enzymes (*Cfol*, *Alul*, and *HaelII*). The differentiation between *Photorhabdus* and *Xenorhabdus* required 3 restriction enzymes (*Cfol*, *Alul*, and *Hinfl* or *Ddel* or *HaeIII* or *Mspl*) (Brunel, Givaudan, Lanois, Akhurst, & Boemare, 1997).

Ribotyping techniques require many restriction enzymes and inability to discriminate all genus and species of *Photorhabdus* and *Xenorhabdus*. However, ribotyping techniques may not be suitable tool (Brunel et al., 1997; Fischer-Le Saux et al., 1999; Lengyel et al., 2005).

4.2.3 DNA sequencing

The discrimination between *Xenorhabdus* and *Photorhabdus* by nucleotide sequencing is the analysis of DNA sequences of the housekeeping genes. In 1991, the complete 16S rDNA sequence was used to distinguish *P. luminescens*, *P. temperate*, and *P. asymbiotica* (Fischer-Le Saux et al., 1999). However, 16S rDNA sequence was not reliable in subspecies level (Akhurst et al., 2004). Later, sequencing of the 16S rDNA, 50S ribosomal protein L2 (*rplB*) gene, recombinase A (*recA*), DNA gyrase beta subunit (*gyrB*), DNA polymerase III subunit beta (*dnaN*), and glutamyl-tRNA synthetase (*gltX*) were used to distinguish between *Photorhabdus* and *Xenorhabdus* species. The *recA*, *gyrB*, *dnaN*, and *gltX* sequences contain higher variation than the sequences of *rplB* and 16S rDNA. Moreover, *gyrB* and *rplB* cannot

differentiate all *Photorhabdus* and *Xenorhabdus* species. The *gltX* gene was probable obtained by lateral gene transfer, therefore, it not suitable tool for differentiation. The phylogenetic relationship of *dnaN* and *recA* provided more correct clustering. Therefore, phylogenetic constructs using individual genes such as *dnaN* or *recA* may provide a sufficient discrimination for *Photorhabdus* and *Xenorhabdus* species (Tailliez et al., 2010).

5. Secondary metabolites

Photorhabdus and *Xenorhabdus* bacteria have ability to invade insect larvae leading insect larvae die by septicemia within 24-48 h. The insect cadavers are slowly decaying because of secondary metabolites, which bacteria released, can inhibit other microorganisms (Koppenhöfer , & Gaugler, 2009). *Photorhabdus* and *Xenorhabdus* produce various secondary metabolites (Figure 10-11).

Benzylideneacetone was produced by *X. nemotaphila* and *X. bovienii*. It has small molecule and heat resistant. This substance used in the food and beverage industry. It has antibiotic activity against Gram-negative bacteria and act as phospholipase A2 inhibitor that relate to Immunosuppression of insect larvae (Ji et al., 2004). Furthermore, these bacteria can produce iodinine (Fodor et al., 2007) phenethylamides and derivative of iodinine (Li, Chen, Webster, & Czyzewska, 1995; McInerney, Gregson, et al., 1991). Xenorhabdins, Xenorxides (Li, Chen, & Webster, 1996) and Xenocoumacins (XCNs) (McInerney, Taylor, Lacey, Akhurst, & Gregson, 1991) were produced by *Xenorhabdus* spp. *X. nemotaphila* can produce XCNs, Xenocoumacin 1 (XCN1) and Xenocoumacin 2 (XCN2), which belong to the antibiotic group. Both XCN1 and XCN2 are antibacterial and antiulcer agents. Moreover, XCN1 are also antifungal agent. *X. nemotaphila* can produce other compounds such as Xenematide and Xenortides which are insecticide (Lang, Kalvelage, Peters, Wiese, & Imhoff, 2008; McInerney, Taylor, et al., 1991).

Photorhabdus bacteria produce secondary metabolites that similar to compounds produced by Gram-positive bacteria. This bacterium produces compounds such as carbapenems, photobactin, 2-isopropyl-5 - [(E) -2-phenylethenyl] benzene-1, 3-diol (IPS) and 2-ethyl-5 - [(E) -2-phenylethenyl] benzene-1,3-diol (ES) that belonging to stilbenes group and have the ability to inhibit the growth of many bacteria including antimicrobial resistant, i.e. Methicillin-resistant *Staphylococcus*

aureus (MRSA) (Hu, Li, Li, Webster, & Chen, 2006). In general, stilbenes are a metabolite produced from plants. This substance can inhibit the growth of microorganisms including Gram-positive and negative bacteria, and fungus. Besides, stilbenes also inhibit the activity of enzyme phenol oxidase in the immune system of insects (Eleftherianos et al., 2007).

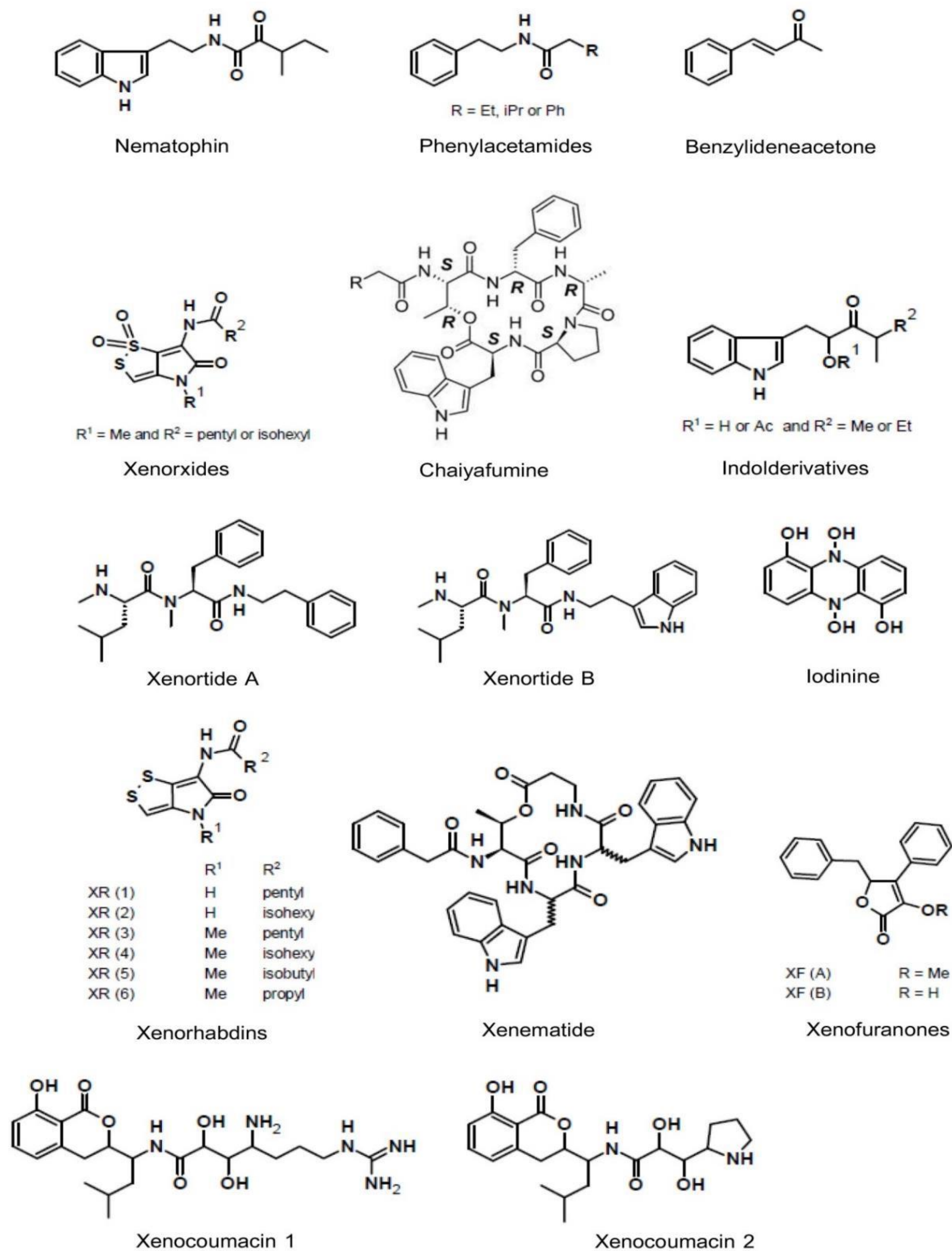

Figure 10 The structure of secondary metabolites were produced by *Xenorhabdus* bacteria

Source: Grundmann et al., 2014

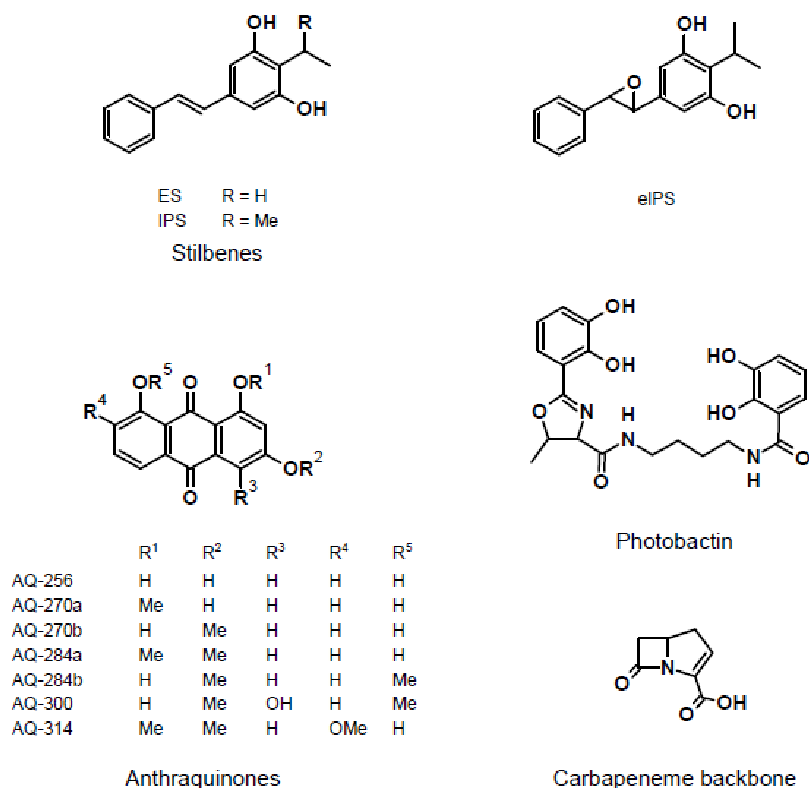

Figure 11 The structure of secondary metabolites were produced by *Photorhabdus* bacteria

Source: Brachmann, 2009

6. Phylogenetic relationship of *Photorhabdus* and *Xenorhabdus*

In 2010, Tailliez et al. studied about phylogenetic relationship of *Photorhabdus* and *Xenorhabdus* bacteria with neighbor-joining analysis of *recA*, *gyrB*, *dnaN*, *gltX* and 16s rDNA. The result showed that the *recA* and *dnaN* sequences provided more correct clustering (Tailliez et al., 2010). In earlier study of symbiotic bacteria in Thailand, Thanwisai et al. analyzed phylogenetic relationship by *recA* gene with maximum-likelihood analysis. *Xenorhabdus* in the country was didived into 2 clusters. The first cluster is related to *X. stockiae* strain TH01. The remaining cluster is related to *X. miraniensis* strain Q1. *Photorhabdus* in Thailand was divided into 3 clusters including the first cluster that related to *P. luminescens* subsp. *akhurstii* strain

FRG04 and *P. luminescens* subsp. *hainanensis* strain C8404, the second cluster related to *P. luminescens* subsp. *laumondii* strain E21, and the last cluster related to *P. asymbiotica* subsp. *australis* strain 9802892 (Thanwisai et al., 2012). Later, *Xenorhabdus* isolates in the upper north of Thailand were closely related to *X. stockiae* strain TH01, *X. stockiae* strain 858516, *X. miraniensis* strain Q1 and *X. ehlersii* strain DSM16337. *Photorhabdus* isolates in the upper north of Thailand are closely related to *P. luminescens* subsp. *akhurstii* strain FRG04, *P. luminescens* subsp. *hainanensis* strain C8404 and *P. luminescens* subsp. *laumondii* strain E21 (Fukruksa, 2014). In the same years, Yimthin reported *Xenorhabdus* in northeastern Thailand are related to *X. stockiae* strain TH01, *X. stockiae* strain 858516, *X. indica* strain DSM17382, *X. ehlersii* strain KR02 and *X. ishibashii* while *Photorhabdus* in this region are related to *P. luminescens* subsp. *akhurstii* strain FRG04, *P. luminescens* subsp. *hainanensis* strain C8404, and *P. asymbiotica* subsp. *australis* (Yimthin, 2014). Moreover, phylogenetic relationship of *Photorhabdus* and *Xenorhabdus* bacteria in 4 provinces of Thailand including Phitsanulok, Chaiyaphum, Nakhon Ratchasima, and Saraburi were evaluated. *Xenorhabdus* isolated are related to *X. stockiae* strain TH01 and *X. stockiae* strain 858516 while *Photorhabdus* are related to *P. luminescens* subsp. *akhurstii* strain FRG04, *P. luminescens* subsp. *hainanensis* strain C8404, *P. luminescens* subsp. *laumondii* strain E21 and *P. asymbiotica* subsp. *australis* strain 9802892 (Suwannaroj, 2014). In addition, the evolutionary relationship of symbiotic bacteria was analyzed in the national park of Thailand. In 2017, Muangpat reported symbiotic bacteria that isolated from EPNs in Mae Wong National Park, Kamphaeng Phet Province were closely related to *X. stockiae*, *X. japonica*, *P. luminescens* subsp. *akhurstii* and *P. temperata* subsp. *temperate* (Muangpat et al., 2017). Then, Yooyangket reported symbiotic bacteria in Nam Nao National Park, Phetchabun Province were closely related to *X. stockiae* strain TH01, *X. vietnamensis*, *X. japonica* and *P. luminescens* subsp. *akhurstii* strain FRG04 (Yooyangket et al., 2018).

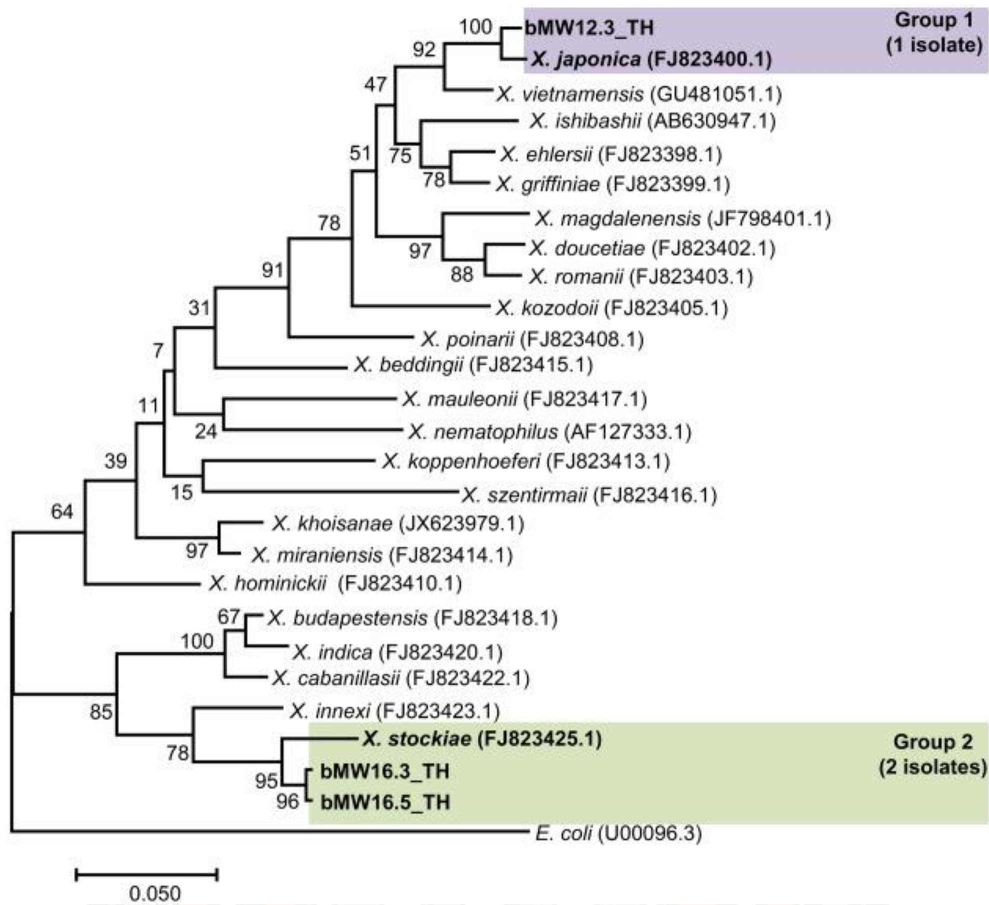

Figure 12 Maximum likelihood tree for *Xenorhabdus*. The phylogenetic tree was based on a region of *recA* (588 bp) for 3 *Xenorhabdus* isolates from Mae Wong National Park, Kamphaeng Phet Province, Thailand grouped with 23 sequences of the *Xenorhabdus recA* gene downloaded from NCBI

Source: Muangpat et al., 2017

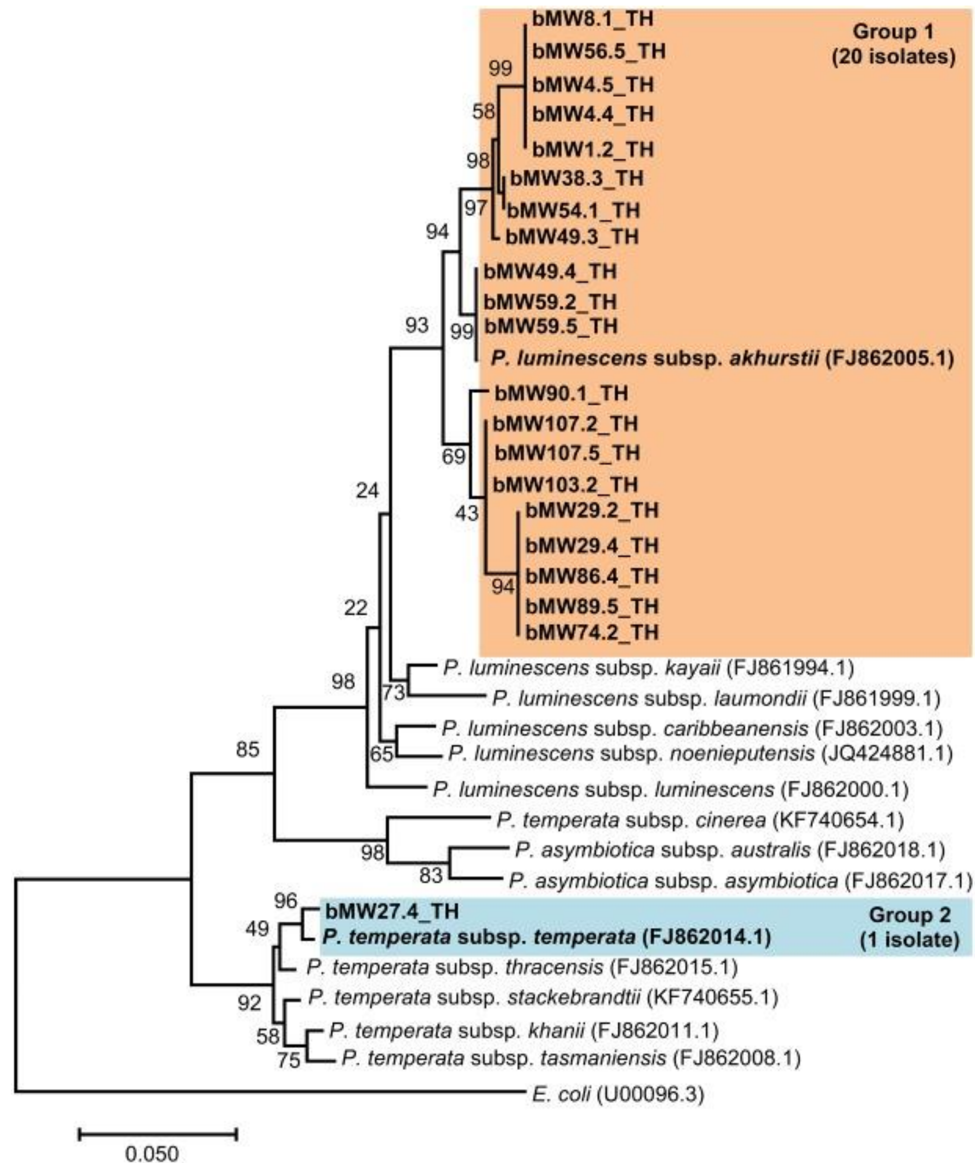

Figure 13 Maximum likelihood tree for *Photorhabdus* The phylogenetic tree was based on the partial recA gene (588 bp) for 21 *Photorhabdus* isolated from Mae Wong National Park, Kamphaeng Phet Province, Thailand grouped with 14 sequences of the *Photorhabdus recA* gene downloaded from NCBI

Source: Muangpat et al., 2017

7. Application for controlling medical insects

Photorhabdus and *Xenorhabdus* are causing insect death (Bode, 2009; Ruiu, Satta, & Floris, 2013). Both bacteria live within the intestine of EPNs which enter to haemocoel of insect and release symbiotic bacteria into hemolymph. Then, insect host die within 24-48 h by septicemia. Several applications of symbiotic bacteria on control of insect pest have been reported. Therefore, *Photorhabdus* and *Xenorhabdus* were used as a pesticide. The efficiency of *P. luminescens* with *Bacillus thuringiensis* can control *Spodoptera littoralis* when compare with the control group (Benfarhat-Touzri et al., 2014).

In the same way, *Photorhabdus and Xenorhabdus* have been reported for control mosquito. Silva and collaborator evaluated the efficiency of *Photorhabdus and Xenorhabdus* against *Ae. aegypti* in a 3-4-day period. It was found that *P. luminescens* killed up to 73% of *Ae. aegypti* in the feed group, and 83% in unfed group, while, *X. nematophila* can kill 52% of *Ae. aegypti* in the feed group and 42% in unfed group (da Silva et al., 2013). Furthermore, Ullah and collaborator reported *P. temperate* M1021 and *P. luminescens* TT01 are capable of producing 4 toxic insecticides including Toxin complexes (TCs), *Photorhabdus* insectrelated (Pir) proteins, Makes Caterpillars Floppy (Mcf) toxin, and *Photorhabdus* virulence cassettes (PVC). The Mcf toxin from *P. temperate* M1021 lead to insect larva death with wrinkle skin due to the destruction of midgut mucosa (Ullah et al., 2014). In 2015, Park et al. evaluated the larvicidal activity of Cry4Ba toxin from *Bacillus thuringiensis* synergists with *Photorhabdus and Xenorhabdus* bacteria to control *Ae. aegypti.* The result showed larvicidal activity of *X. nematophila* synergists with Cry4Ba toxin and *P. luminescens* synergists with Cry4Ba toxin was 95% and 85%, respectively. The reason that casing lavae death are symbiotic bacteria, *Photorhabdus* and *Xenorhabdus*, lead to septicemia. The Cry4Ba toxin synergists with *Photorhabdus* and *Xenorhabdus* bacteria have ability to pore formation in midgut induce substance imbalance, then, *Ae. aegypti* larva stop feeding, paralyze and die (Park, 2015).

In Thailand, several reports about *Aedes* larva control by *Photorhabdus and Xenorhabdus* bacteria have been reported. In 2014, Fukruksa and collaborators evaluated the efficiency of *Photorhabdus and Xenorhabdus* in upper north of

Thailand for control *Ae. aegypti* larvae. The result showed *Xenorhabdus ehlersii* bMH9.2_TH has high efficiency as 100% at 96 h (Fukruksa, 2014) (Fukruksa, 2014). Then, in 2018, Yooyangket and collaborator revealed that *X. stockiae* bNN112.3_TH had highest efficacy against *Ae. aegypti* larvae having 99-100% after 96 h exposure in both fed and unfed group. *Photorhabdus luminescens* subsp. *akhurstii* bNN61.4_TH and *P. luminescens* subsp. *akhurstii* bNN121.4_TH showed highest efficacy against *Ae. albopictus* larvae having 83-100% after 96 h exposure in both fed and unfed group (Yooyangket et al., 2018).

Relationship of entomopathogenic nematodes and their symbiotic bacteria

Entomopathogenic nematodes belonging to the families Heterorhabditidae and Steinernematidae have symbiotic associations with bacteria from the *Photorhabdus* and *Xenorhabdus* genera, respectively.. Entomopathogenic nematodes are worldwide distribution in the soil of several environments. At present, 28 heterorhabditid and 100 steinernematid nematodes have been described. While, 5 species with 19 subspecies of *Photorhabdus* and 26 species of *Xenorhabdus* have been described. Each EPN species maintains a mutualistic association with a single species of symbiotic bacteria. However, each symbiotic bacteria species serves as a symbiont for multiple EPN species (Table 6) (Koppenhöfer , & Gaugler, 2009; Yimthin, 2014).

Table 6 List of entomopathogenic nematodes and their symbiotic bacteria

Symbiotic bacteria	Entomopathogenic nematode
P. asymbiotica subsp. *asymbiotica*	*H. indica*
P. asymbiotica subsp. *australis*	*H. gerrardi* *H. indica*
P. heterorhabditis	*H. zealandica*
P. luminescens subsp. *akhurstii*	*H. baujardi* *H. indica*
P. luminescens subsp. *caribbeanensis*	*H. bacteriophora*
P. luminescens subsp. *hainanensis*	*H. bacteriophora*
P. luminescens subsp. *kayaii*	*H. bacteriophora*
P. luminescens subsp. *kleinii*	*H. heliothidis* *H. megidis*
P. luminescens subsp. *laumondii*	*H. bacteriophora*
P. luminescens subsp. *luminescens*	*H. bacteriophora*
P. luminescens subsp. *sonorensis*	*H. sonorensis*
P. luminescens subsp. *thracensis*	*H. bacteriophora*
P. temperata subsp. *cinerea*	*H. downesi* *H. megidis*
P. temperata subsp. *khanii*	*H. heliothidis* *H. megidis*
P. temperata subsp. *stackebrandtii*	*H. bacteriophora*
P. temperata subsp. *tasmaniensis*	*H. marelatum* *H. zealandica*
P. temperata subsp. *temperata*	*H. megidis*
P. zealandica	*H. zealandica*
X. beddingii	*S. longicaudum*

Symbiotic bacteria	Entomopathogenic nematode
X. bovienii	*S. affine*
	S. ceratophorum
	S. feltiae
	S. intermedium
	S. jollieti
	S. kraussei
	S. litorale
	S. oregonense
	S. puntauvense
	S. sichuanense
	S. weiseri
X. budapestensis	*S. bicornutum*
	S. ceratophorum
X. cabanillasii	*S. riobrave*
X. doucetiae	*S. diaprepesi*
X. ehlersii	*S. longicaudum*
	S. serratum
X. griffiniae	*S. hermaphroditum*
X. hominickii	*S. arenarium*
	S. karii
	S. monticolum
X. indica	*S. thermophilum*
	S. abbasi
X. innexi	*S. scapterisci*
X. Ishibashii	*S. aciari*
X. japonica	*S. kushidai*
X. khoisanae	*S. khoisanae*
X. koppenhoeferi	*S. scarabaei*

Symbiotic bacteria	Entomopathogenic nematode
X. kozodoii	*S. aquliae*
	S. arenarium
X. magdalenensis	*S. austral*
X. miraniensis	*S. khoisanae*
X. namatophila	*S. carpocapsae*
	S. feltiae
X. namatophila	*S. websteri*
X. poinarii	*S. cubanum*
	S. glaseri
X. romanii	*S. puertoricense*
X. stockiae	*S. siamkayai*
	S. websteri
X. szentirmaii	*S. rarum*
X. vietnamensis	*S. sangi*

***Aedes* mosqutioes**

1. *Aedes albopictus*

Aedes (*Stegomyia*) *albopictus*, also known as Asian tiger mosquito is classified in phylum Arthropoda, class Insecta, order Diptera and family Culicidae. This mosquito undergoes complete metamorphosis with 4 stage including egg, larvae, pupa and adult. The adults have white or silver scale patches on black body and legs look like black and white pattern. The presence of a median silver-scale line against a black background on the scutum is a dominant feature of this species. (Figure 14) Adult females usually bite during the day at outdoor areas (Genchi, Rinaldi, Mortarino, Genchi, & Cringoli, 2009), in the same way as breeding site. *Ae. albopictus* associated with to arboviruses and considerate to the effective vector of human disease such as Chikungunya, Dengue fever and Zika (Gratz, 2004).

2. *Aedes aegypti*

Aedes (*Stegomyia*) *agypti* or yellow fever mosquito is classified in Phylum Arthropoda, Class Insecta, Order Diptera and Family Culicidae. It is vector of several viruses including Dengue virus, Chikungunya virus and yellow fever virus. The scutum of *Ae. aegypti* has a characteristic like a lyre shaped marking of white scales and also has a pair of submedian-longitudinal white lines (Figure 14). *Ae. aegypti* are native mosquito in Africa. It is currently found in tropical and subtropical regions around the world. The life cycle of *Ae. aegypti* is complete metamorphosis that has four stages including egg, larva, pupa and adult (Diagne et al., 2015; Gratz, 2004)

Ae. aegypti also has limited dispersal capability because of the flight range is only 200 m (Turell et al., 2005). The breeding and resting sites of this species usually are artificial water at indoors rather than natural water (Jansen, & Beebe, 2010).

Figure 14 Morphology of adult stage *Aedes albopictus* (left) and *Aedes aegypti* (right)

Source: Gathany, 2007a, 2007b

3. The biology and life cycle of *Aedes albopictus* and *Aedes aegypti*

The female mosquito lay eggs on damp surfaces and temporarily flood. Oviposition site of *Aedes albopictus* and *Aedes aegypti* are different. *Aedes albopictus* usually deposit their eggs in natural water or agricultural area such as tree holes, bamboo stem and orchard. On the other hand, the oviposition site of *Ae. aegypti* is

artificial water at indoors or human habitat area such as flower vase and plant saucers. *Aedes* lay their eggs separately and spread out the eggs over several sites. The eggs are ovoid shaped, long, smooth and 1 millimeter long. The newly eggs have white color and change to black color within 12-24 h later. The eggs can be placed in a dry environment for a long time and hatch as larvae when placed in water for 3-4 days

After eggs hatch, larvae start feeding and molting to develop for 4 times (L1, L2, L3 and L4) within 5-8 days. In the last molting, larvae develop to pupae. Mosquito pupae exhibit mobility and responsiveness to stimuli. Unlike larvae, pupae do not engage in feeding activities and undergo a developmental transition to adults over a span of approximately two days.

Upon reaching maturity, adult mosquitoes emerge by ingesting air to expand their abdomen, causing the pupal case to split open. They then emerge from the case headfirst. Adult start to breeding at the age of 24 hrs. Female adult breeding only once in its life cycle but lay eggs for several times. Female adults usually bite during the daytime. After eating blood, the female mosquitoes rest and wait for eggs development, gonotrophic cycle, which takes 3 - 4 days. The resting site of this mosquito is dark and windless areas especially toilet or hanging objects inside the house such as clothes, mosquito net and awning. After the eggs are fully mature, the females find the place to lay egg (Jia et al., 2016).

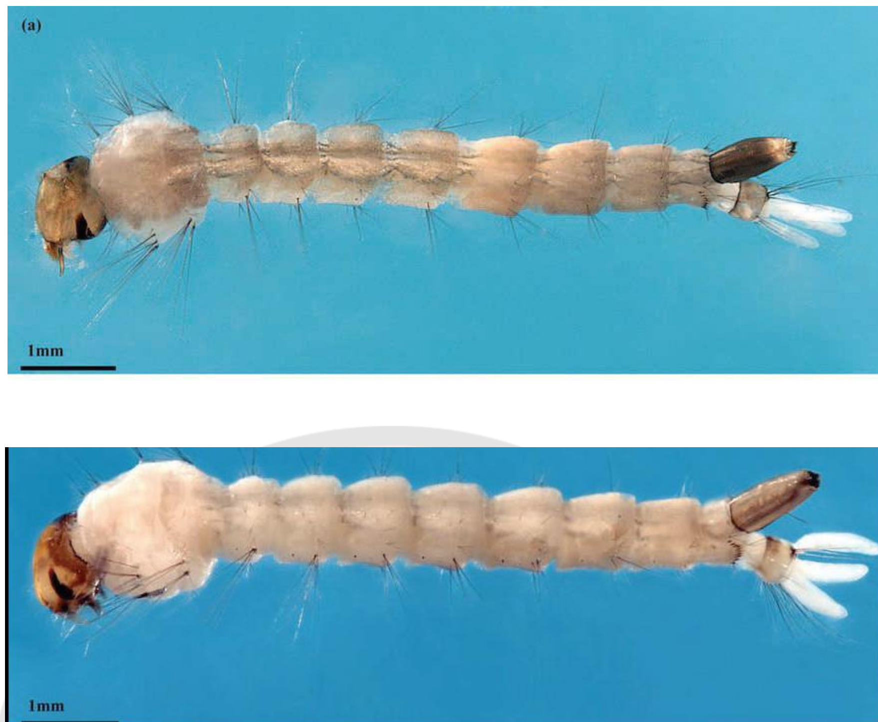

Figure 15 The larva stage of *Aedes albopictus* (up) and *Aedes aegypti* (down)

Source: Farajollahi, & Price, 2013

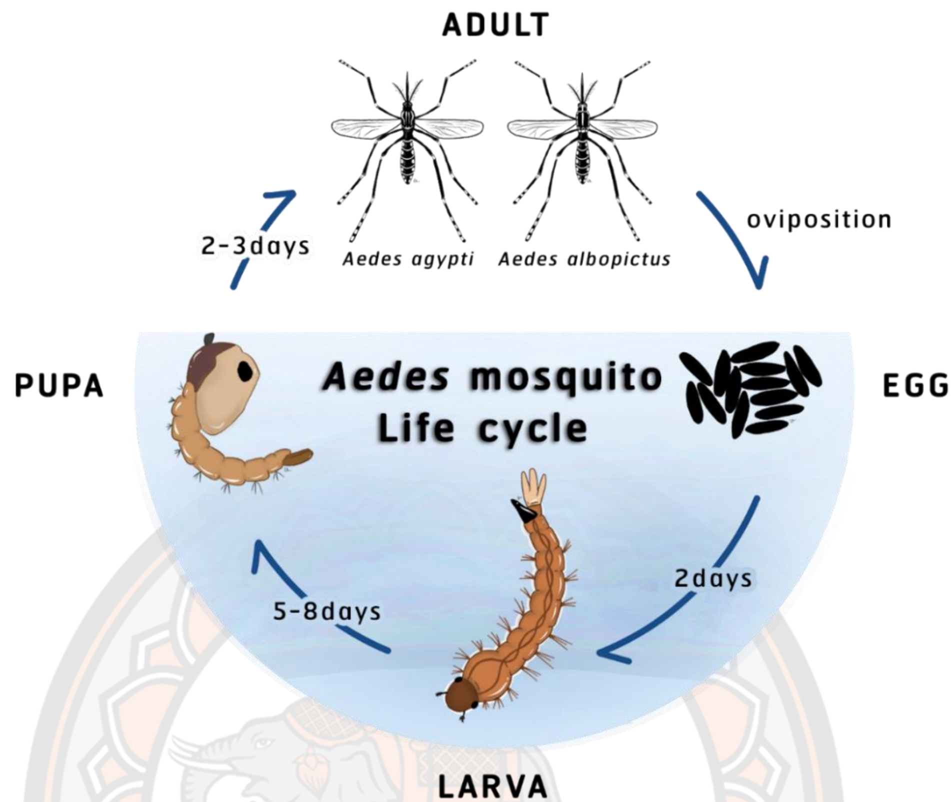

Figure 16 Life cycle of *Aedes* mosquitoes

4. Mosquito control

Preventing or reducing mosquito vectors help to control of human–vector contact that reduce opportunity of infection. Base on the WHO recommendation, control strategies of *Aedes* are the using of combination of three methods, environmental management, chemical control, and biological control. Furthermore, interested alternative method is genetic control (World Health Organization, 2020b).

Environmental management is simple method for controlling mosquitoes by disposing of, modifying, eliminating, or repurposing non-essential containers. that provide immature mosquito (egg, larva and pupa) habitats. Improvement of water supply, water-storage systems and installation of reliable piped water are a basic method of controlling *Aedes* vectors, especially *Ae. aegypti.* Water-storage containers can be engineered to deter mosquito egg deposition on the water surface. These containers can be securely sealed with tight-fitting lids. The utmost priority is changing the human residential environment or behavior such as installing mosquito

screening on windows and using mosquito nets while sleeping during daytime hours (World Health Organization, 2020b).

Chemical control is method that use various chemical agents for controlling mosquitoes both larva (larvicide) and adult (adulticides) stage. Organophosphates as temephos or fenthion are the most common chemical agent for using as pesticide. Moreover, juvenoids or juvenile hormones such as methoprene and diflubenzuron also use for inhibit insect development. These substances cause insect larva death or grow abnormally. It is common agent to control mosquitoes in the larvae stage (Lucia, Harburguer, Licastro, Zerba, & Masuh, 2009; Salokhe, Deshpande, & Mukherjee, 2012; Silva , & Mendes, 2007). Futhermore, pyrethroids are the one of synthetic insecticides that have been used to control mosquito. It disrupts nervous system of insect lead to insect die. However, using the chemical agent for a long time, the mosquito will resistant to those chemical agents and toxic to environment (Prophiro et al., 2011). Amelia-Yap and co-authors reviewed various researches about pyrethroid resistance in *Ae. aegypti* in Southeast Asia including Thailand. They revealed that *Ae. aegypti* in this area developed to resistance of pyrethroid. Adult of *Ae. aegypti* elevated tolerance or resistance to different types of pyrethroids, for example deltamethrin, permethrin, dieldrin, bioallethrin, bioresmethrin or alphacypermethrin (Amelia-Yap, Chen, Sofian-Azirun, & Low, 2018).

Biological control is the method that control of mosquitoes by using organisms or microorganisms that are present in the environment. Larvivorous fish species such as Guppy (*Poecilia reticulata*), also known as millionfish and rainbow fish, and predatory copepods (small freshwater crustaceans) are effective against immature larval stages of mosquitoes. *Bacillus thuringiensis* subsp. *israelensis* and *B. sphericus* (Kovendan, Murugan, Vincent, & Barnard, 2012), and insect fungi, *Metarhizium anisopliae* (Leles, D'Alessandro, & Luz, 2012) were have been report as biocontrol agents for control mosquitoes. Moreover, bacteria in genus *Photorhabdus* and *Xenorhabdus* has the ability to control insects. Both bacteria produce various secondary metabolites that act as insecticidal compounds. The bacteria enter to the hemolymph of insect by EPNs that penetrate insect host through natural opening. Then, EPNs release symbiotic bacteria form their intestine. The symbiosis bacteria

multiply and release toxic metabolite to insect hemolymph that cause insect death within 48 h (Forst et al., 1997; Goodrich-Blair , & Clarke, 2007). Previous studies showed that *X. ehlersii* bMH9.2_TH and *P. luminescens* exhibit the highest potential as a pathogen against the *Ae. aegypti* larvae. *Xenorhabdus ehlersii* bMH9.2_TH demonstrated complete efficacy in larval mortality within 96 hours, whereas *P. luminescens* achieved efficiency levels of 73-83% within 24 hours (da Silva et al., 2013; Fukruksa et al., 2017).

Genetic control represents an innovative approach to managing *Aedes* mosquito populations. This strategy is categorized based on its intended outcome: suppression or replacement. Population suppression strategies aim to minimize the vector count within a specific population or area, akin to traditional insecticide-based method. On the other hand, population replacement strategies seek to diminish the vectorial capacity of mosquitoes within the target population. For instance, the objective of sterile-male methods is to inhibit target populations. For instance, sterile-male techniques are designed for population suppression. In this approach, genetically modified sterile males are released to mate with wild-type females. The genetic modification causes the offspring of these mating to experience reduced viability or even death. By releasing a sufficient number of sterile males over a sustained period, the target population can be suppressed, potentially leading to its elimination. Conversely, population replacement strategies are intended to mitigate the risk of niche replacement, wherein the removal of one pest leads to its replacement by another species. In the context of genetic control, population replacement aims to decrease the vectorial capacity of mosquitoes, minimizing the potential for niche replacement dynamics to occur (Alphey, 2014).

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CHAPTER III

RESEARCH PROCEDURES OF THE STUDY

Soil sample collection

In this study, soil samples in 12 provinces of Thailand including Chiang Mai, Kalasin, Khon Kaen, Lopburi, Mae Hong Son, Nan, Phayao, Phetchaburi, Phrae, Sakon Nakhon, Saraburi and Uttaradit were randomly collected. In an area, 500 grams from each 5 soil samples were collected using hand shovel with 10-centimeter depth. Soil sample was kept individually in plastic bag for preventing from losing moisture. Then, extrinsic factors including soil temperature, Soil pH and moisture were recorded with soil survey instrument AMT-300 (Model: KC-300B, Yanchang Kecheng Optoelectronic Technology Co., Ltd, China) and Soil pH and moisture tester (Modal DM-15, Takemura electric works, Ltd, Japan). Moreover, geographic coordinates including latitude, longitude, meters above mean sea level were recorded by using Garmin nuvi 1250 GPS navigator (Garmin, Taiwan). All soil samples were transferred to department of Microbiology and Parasitology, faculty of Medical Science, Naresuan University, Phitsanulok province, Thailand.

Isolation of entomopathogenic nematodes from soil sample

Entomopathogenic nematodes were isolated from soil sample by baiting technique (Bedding, & Akhurst, 1975). The waxworm (*Galleria mellonella*) larvae were used as baits. Each soil sample was placed in plastic box with five *G. mellonella* larvae. The box was stood for 5 days. The dead larvae were observed and collected for EPNs isolation. Then, re-baiting technique with five new *G. mellonella* larvae was performed to maximize the EPN.

Isolation of entomopathogenic nematodes from *G. mellonella* larvae

Entomopathogenic nematodes were isolated from dead *G. mellonella* larvae by White trap technique. This trap used 2 pieces of plastic petri dish. The small size (60 x 15 mm) inversely places in the large size (100 x 15 mm) and place long filter

paper in the middle of small dish like a bridge. Sterile distilled water (DW) was added in large dish. Then, the dead *G. mellonella* larvae were placed on the filter paper. The plates were incubated at dark for 15-20 days in room temperature. The infective juvenile (IJ) stage of EPNs were observed by stereo microscope. The IJs were collected, cleaned with sterile distilled water, and stored in culture flask at 13ºC refrigerator for 2-3 months. In addition, five microcentrifuge tubes containing approximately 200 IJs each were kept at -20ºC for DNA extraction.

Propagation and storage of entomopathogenic nematodes

Propagation of EPNs was done for increasing the number of EPNs which use in larvicidal activity study. Five *G. mellonella* larvae were placed in plastic petri dish (30 x 15 mm). A few drops of the IJs suspension were transferred to *G. mellonella* larvae. The petri dish was sealed with paraffin film. The plate was incubated at room temperature for 2-4 days. Dead *G. mellonella* larvae were observed daily. The dead larvae were subjected to White trap. The emerged EPNs were collected and kept in 50-milliliter (ml) culture flask. EPNs were cleaned with sterile DW for several times. Finally, the culture flask containing EPNs with approximately 5-10 ml was stored in 13ºC refrigerator.

Identification of entomopathogenic nematodes

1. DNA extraction of EPNs

The genomic DNA of EPNs was extracted with Phire Tissue Direct PCR Mastermix kit (Thermo scientific, USA) as recommendation by the manufacturer. An approximately 200-500 IJs which kept in microcentrifuge tube at -20ºC were mixed with 20 µl dilution buffer. Then, 0.5 µl DNA release was added. EPNs were homogeneously grinded with 1 ml sterile tip. The tube was spin down and incubated at 95 ºC in water bath for 5 min. The tube was centrifuged at 12,000 g for 5 min. The supernatant containing genomic DNA was transfer to new 1.5 µL centrifuge tube. The 1 µL of collected supernatant were analysed for checking DNA quantity by running on 0.8% agarose gel electrophoresis (100 volte, 30 minutes). The gel was stained with Ethidium bromide (EtBr), de-stained with distilled water, and visualized the genomic

DNA band under UV light. The supernatant (genomic DNA) was used for polymerase chain reaction.

2. Polymerase chain reaction (PCR) for EPNs

Polymerase chain reaction was performed using Phire Tissue Direct PCR Mastermix kit (Thermo scientific, USA) in a thermal cycler (Applied Biosystems, Pittsburgh, PA, USA). A partial fragment of the ITS (1,000-1,100 bp) and 28S rDNA (870 bp) regions were amplified for *Heterorhabditis* and *Steinernema*. The used nucleotide primers show in Table 7. The components of PCR reagent and thermal cycling show in Table 8 and 9 respectively. The amplified PCR products were analysed by running on 1.2% agarose gel electrophoresis (100 volte, 30 minutes). The gel was stained with Ethidium bromide (EtBr), de-stained with distilled water, and visualized the DNA band under UV light.

Table 7 List of primer for identification of *Steinernema* and *Heterorhabditis*

Gene	Primer
28S	539_F (5'GGATTTCCTTAGTAACTGCGAGTG-3') 535_R (5'-TAGTCTTTCGCCCCTATACCCTT-3')
ITS	18S_F (5'- TTGATTACGTCCCTGCCCTTT-3') AB28_R (5'- ATATGCTTAAGTTCAGCGGGT-3')

Table 8 Components of PCR reagent for amplification of *Steinernema* and *Heterorhabditis*

Reagent (concentration)	Volume (µL)
Phire Tissue MasterMix (2X)	15
Forward primer (5 µM)	1.2
Reward primer (5 µM)	1.2
DNA (20-200 ng)	1.8
Distilled water	10.8
Amount	30

Table 9 Thermal cycling for amplification of entomopathogenic nematodes

Parameter	Temperature		Time	
Initial denature	98ºC		5 min	
Denature	98ºC		5 sec	30 cycles
Annealing	*Steinernema* : 55ºC	*Heterorhabditis* : 50ºC	5 sec	
Extension	72ºC		30 sec	
Final-extension	72ºC		1 min	

3. DNA purification and sequencing for entomopathogenic nematodes

Purification of amplified PCR was performed according to NucleoSpin® Gel and PCR Clean-up as manufacturer instruction. The PCR products of 29 µl was mixed with 58 µl of NTI reagent. The mixture containing DNA sample was loaded onto nucleoSpin® Gel and PCR Clean-up column placed on a collection tube (2 ml). The tube was centrifuged at 11,000 g for 30 sec. The flow-through was discarded and the column was put back into the collecting tube. Seven-hundred microliters of the buffer NT3 were added to the NucleoSpin® Gel and PCR Clean-up Column. The tube was centrifuged 11,000 g for 30 sec. The flow-through was discarded and the column was put back into the collection tube. To remove buffer NT3 completely, the tube was centrifuged at 11,000 x g for 1 min. The NucleoSpin® Gel and PCR Clean-up Column was transferred into a new 1.5 ml microcentrifuge tube. Twenty microliters of sterile water were added. The tube was incubated at room temperature for 1 min and centrifuged at 11,000 g for 1 min. The purified PCR products were checked by running 1 µl on 1.2% agarose gel electrophoresis (100 volte, 30 minutes). The agarose gel was stained with ethidium bromide solution and de-stained with distilled water. The band of the purified PCR fragment was observed under UV light. The purified PCR products were sent to South Korea for sequencing at Macrogen, Inc.

4. Sequence analysis of entomopathogenic nematodes

The sequences of ITS and 28S rDNA were edited with SeqManII (DNASTAR inc., Wisconsin, USA). The edited sequences were subjected to the BLASTN program to compare with known sequences in the NCBI database. A cut-off of 97% identity was considered for the same species.

5. Phylogenetic analysis

Alignment of the nucleotide sequences in this study with known sequences were analysed by ClustalW. Phylogenetic tree was constructed based on the neighbour-joining (NJ) method (Kimura-2-parameter, Bootstrap = 1,000) and the maximum likelihood (ML) method (Tamura-Nei-parameter, Bootstrap = 1,000) with the MEGA program Version 7.0 (Kumar, Stecher, & Tamura, 2016) Besides, Bayesian Inference (BI) of phylogeny was constructed by the MrBayes program (Ronquist et al., 2012).

Isolation of *Photorhabdus* and *Xenorhabdus* from entomopathogenic nematodes

Symbiotic bacteria were extractedfrom the hemolymph of deceased *G. mellonella* larvae that had been infected with the IJs of EPNs. The dead larvae of *G. mellonella* were cleaned by dipping into absolute ethanol for 1 min followed by air-drying on a sterile petri dish. Subsequently, the third segment from the head of the *G. mellonella* larvae was carefully torn using sterile forceps. The hemolymph on the teared area of dead larvae were transferred by sterile loop and streaked on nutrient agar supplemented with bromothymol blue and triphenyl-2,3,5-tetrazolium chloride (NBTA). The plate was incubated at dark in room temperature for 4 days. Preliminary identification of colony morphology was performed based on color of colony which *Photorhabdus* has green color and *Xenorhabdus* has blue colony (Thanwisai et al., 2012).

Identification of *Photorhabdus* and *Xenorhabdus*

1. DNA extraction of symbiotic bacteria

A single colony of each isolate of symbiotic bacteria was inoculated in 15 ml centrifuge tube containing 5 ml of Luria-Bertani (LB) broth. The tube was incubated overnight at room temperature and centrifuged at 150 rpm (18-24 h). Then,

the tube was centrifuged at 12,000 g for 1 min to obtain the bacterial pellets. The genomic DNA of symbiotic bacteria was extracted from bacterial pellets using the Genomic DNA Mini Kit (Blood/Cultured Cell) (Geneaid Biotech Ltd., Taiwan). Bacterial pellets were mixed with GB 200 µl then shake vigorously and incubate in room temperature for 5 min. After that, Proteinase K 20 µl was added and incubate in 60ºC for 8 min. Then, GB buffer 200 µl was added and incubated in 70ºC for 10 min. In the same time pre-heated elution buffer 50 µl/sample was prepared by incubating in same temperature as above. Next, absolute ethanol 200 µl was added and transferred to GD column. The column with sample was centrifuged at 12,000 g for 2 min, discarded the collection tube and placed the GD column in a new collection tube. W1 Buffer 400 µl was added to the GD column then centrifuge at 12,000 g for 30 seconds. The flow-through was disposed of, and placed back in same collection tube. Wash Buffer 600 µl with ethanol was added to the column and centrifuge at 12,000 g for 30 seconds then discard the flow-through and place the column back in same collection tube. The column was centrifuged at 12,000 g for 3 min to dry the column matrix. The dried GD Column was transferred to a clean 1.5 ml microcentrifuge tube then 50 µl pre-heated elution buffer will be added. Let column with 1.5 ml microcentrifuge tube stand for at least 3 min to ensure the elution buffer is completely absorbed. Last, the column was centrifuged at 12,000 g for 30 seconds to elute the purified DNA. The purified genomic DNA was analysed for checking DNA quantity by running on 0.8% agarose gel electrophoresis (100 volte, 30 minutes). The gel was stained with Ethidium bromide (EtBr), de-stained with distilled water, and visualized the genomic DNA band under UV light. The genomic DNA of symbiotic bacteria was stored at -20ºC for using as the DNA template in PCR.

2. Polymerase chain reaction for symbiotic bacteria

PCR was performed using EconoTaq® PLUS 2X Master Mixes (Lucigen, USA) in a thermal cycler (Applied Biosystems, Pittsburgh, PA, USA). A partial *recA* (890 bp) fragment of *Photorhabdus* and *Xenorhabdus* was amplified by using a pair of primers; recA_F (5’-GCTATTGATGAAAATAAACA-3’) and recA_R (5’–RATTTTRTCWCCRTTRTAGCT-3’). In addition, part of the 16S rRNA gene was analyzed when the required *recA* gene region could not be identified. The primers 16SP1_F (5´-GAAGAGTTTGATCATGGCTC -3´) and 16SP2_R (5´-

AAGGAGGTGATCCAGCCGCA -3´) were used for amplify the partial region of the 16S rRNA gene (1,500 bp). The components of PCR reagent, thermal cycling of partial *recA* fragment and thermal cycling of partial 16S rRNA region showed in Table 10, 11 and 12 respectively. The amplified PCR products were analysed by running on 1.2% agarose gel electrophoresis (100 volte, 30 minutes). The gel was stained with Ethidium bromide (EtBr), de-stained with distilled water, and visualized the DNA band under UV light.

Table 10 Components of PCR reagent for amplification of symbiotic bacteria

Reagent (concentration)	Volume (µL)
EconoTaq PLUSMaster Mix (2X)	15
Forward primer (1 µM)	1.5
Reward primer (1 µM)	1.5
DNA (10 ng/ µl)	1.5
Distilled water	10.5
Amount	30

Table 11 Thermal cycling for amplification of partial *recA* fragment

Parameter	Temperature	Time	
Initial denature	94ºC	5 min	
Denature	94ºC	1 min	30 cycles
Annealing	45ºC	45 sec	30 cycles
Extension	72ºC	2 min	30 cycles
Final-extension	72ºC	7 min	

Table 12 Thermal cycling for amplification of partial 16S rRNA region

Parameter	Temperature	Time	
Initial denature	94ºC	2 min	
Denature	95ºC	30 sec	30 cycles
Annealing	55ºC	30 sec	
Extension	72ºC	1 min	
Final-extension	72ºC	7 min	

3. DNA purification and sequencing of symbiotic bacteria

Purification of amplified PCR products of symbiotic bacteria was performed as same as methods used in EPNs with exception of the use of different primers for sequencing.

4. Sequence analysis of symbiotic bacteria

A partially nucleotide sequences of *recA* or 16S rRNA region were edited with SeqManII (DNASTAR inc., Wisconsin, USA). The edited sequence was subjected to BLASTN program from NCBI to find a similarity with known organism sequences. A cut-off of 97% identity was considered to the same species.

5. Phylogenetic analysis of symbiotic bacteria

Alignment of the nucleotide sequences in this study with known sequences was analysed by ClustalW. Phylogenetic tree was analysed based on neighbor-joining (NJ) method (Kimura-2-parameter, bootstrap = 1,000) and maximum likelihood (ML) method (Tamura-Nei-parameter, Bootstrap = 1000) in MEGA 7.0 (Kumar et al., 2016). Additional, Bayesian inference (BI) of the phylogeny was constructed using MrBayes (Ronquist et al., 2012).

Evaluation of entomopathogenic nematodes against *Aedes aegypti* larvae

1. Rearing of *Aedes* larvae

The eggs of *Ae. aegypti* (laboratory strain) on a filter paper were purchased from the Taxonomy and Reference Museum, Department of Medical

Sciences, the National Institute of Health of Thailand, Ministry of Public Health, Thailand.

Aedes eggs were placed into dechlorinate water to facilitate larval hatching. The larvae were nourished with minced pet food. The third and fourth instar larvae were collected to test in this experiment.

2. Preparation of entomopathogenic nematodes

Five EPN isolates including one isolate of *S. surkhetense* (ePYO8.5_TH) and four isolates of *H. indica* (eLBI9.2_TH, eMSN1.2_TH, eMSN4.3_TH, and ePRE6.3_TH) were selected for testing their efficacy on *Ae. aegypti* larvae. The EPNs were propagated in *G. mellonella* larvae. White trap technique was performed as previously described in section isolation of EPNs from *G. mellonella* larvae. Emerged IJs were cleaned with DW several times, collected in a 50-ml culture flask and kept in 13ºC refrigerator until using in bioassay. Within two weeks, the flask containing the IJs were took out of the refrigerator, placed at RT for 30–60 min and transferred to Sedgewick Rafter counting chambers for enumeration.

3. Evaluation of entomopathogenic nematodes against *Aedes aegypti* larvae in 24-well plate

Five selected EPN isolates were exposed to the third and fourth instar larvae of *Ae. aegypti*. Ten larvae of *Ae. aegypti* were added to a 24-well plate (15.6 mm-diameter) in four replicates. The excess water was sucked out with a pipette. Then, 800, 1600, 3200 and 6400 IJs of each EPNs in 2 ml of dechlorinated water were transferred to 24-well plates. The dechlorinated water without EPNs were used as negative control. The plate was incubated at 25–28ºC with a photoperiod of 12 h light and 12 h dark (12L:12D). The dead larvae were observed under stereo microscope at 24, 48, 72 and 96 h after exposure. Only death *Ae. aegypti* larvae with EPN inside their body were counted for mortality rate. Each test was run three times on different days. Mortality rate was calculated from the average of 12 repeats. The larvae that have pupated during the test period were excluded from the analysis. If the pupation rate among the control larvae exceeds 10% during the experiment, the test will be invalidated and subsequently repeated. The mortality rate (percentages) of the larvae in control groups and in treatment group (each EPN isolate) was statistically tested using STATA version 13 (ANOVA, $P<0.05$).

4. Evaluation of entomopathogenic nematodes against *Aedes aegypti* larvae in 6-well plate

The most virulence isolate from 24-well plate studies, *S. surkhetense* (ePYO8.5_TH), was selected for testing in the larger container to observe a constant efficacy. The tests were run in 6-well plates (34.8 mm-diameter) using the same method mentioned above with slight modification. Ten larvae of *Ae. aegypti* were added into a 6-well plate in four replicates. Ten millilitres of dechlorinated water containing 4000, 8000,16000, and 32000 IJs of *S. surkhetense* (ePYO8.5_TH) was added directly to a well of 6-well plate. Dechlorinated water without EPNs was used as control. The plates were incubated at 25–28 ◦C with 12L:12D. The dead larvae were observed at 24, 48, 72, and 96 h after exposure. Only death *Ae. aegypti* larvae with EPN inside their body were counted for mortality rate. Each test was run two times on different days, and the mortality rate was calculated from the average of 8 repeats. The mortality rate (percentages) of the larvae in the control groups and the treatment group were statistically tested using STATA version 13 (ANOVA, $P < 0.05$).

Larvicidal activity of symbiotic bacteria against *Aedes aegypti* and *Aedes albopictus* larvae

1. Rearing of *Aedes* larvae

The eggs of *Ae. aegypti* and *Ae. albopictus* (laboratory strain) on a filter paper were purchased from the Taxonomy and Reference Museum, Department of Medical Sciences, the National Institute of Health of Thailand, Ministry of Public Health, Thailand. The rearing of both *Aedes* larvae were same as above description.

2. Preparation of symbiotic bacteria

Photorhabdus (5 isolates) and *Xenorhabdus* (10 isolates) were selected according to their phylogenetic clade distribution. List of selected bacteria show in Table 13. All selected bacterial isolates were separately grown on NBTA. *Escherichia coli* ATCC 25922 (negative control) was cultured on Tryptone soy agar (TSA). A single colony of each isolate of *Photorhabdus*, *Xenorhabdus* on NBTA and *E. coli* ATCC 25922 on TSA was transferred to a 15 ml tube containing 5 ml of 5YS broth medium (5% yeast extract (w/v), 0.5% NaCl (w/v), 0.05% K_2HPO_4 (w/v),

0.05% $NH_2H_2PO_4$ (w/v), 0.02% $MgSO_4.7H_2O$ (w/v) (Shrestha , & Lee, 2012), then incubated at 28◦C in a shaking incubator (150 rpm) for 24 h. To maximize the number of bacterial cells obtained, one millilitre of bacterial culture was transferred into a 50 ml-centrifuge tube containing 39 ml of 5YS broth, incubated at 28◦C in a shaking incubator (150 rpm) for 48 h. The bacterial suspension was centrifuged at 12,000 g for 10 min. The supernatant was discarded. The bacterial pellet was resuspended in sterile DW. The bacterial suspension was adjusted at OD_{600} to 1.0 (10^8 cells/ml) by spectrophotometer (BECMAN-COUTER Model DU®730, Fullerton, USA) (Fukruksa et al., 2017).

Table 13 List of bacteria in larvicidal activity against *Aedes* larvae

Code	Species
bSRI10.1_TH	*Photorhabdus asymbiotica* subsp. *australis*
bSRI10.2_TH	*Photorhabdus hainanensis*
bPSD40.1_TH	*Photorhabdus luminescens* subsp. *akhurstii*
bCMI13.1_TH	*Photorhabdus luminescens* subsp. *akhurstii*
bSNK16.3_TH	*Photorhabdus luminescens* subsp. *akhurstii*
bKKN2.5_TH	*Xenorhabdus eapokensis*
bMSN3.3_TH	*Xenorhabdus griffiniae*
bLBI9.1_TH	*Xenorhabdus griffiniae*
bKKN10.4_TH	*Xenorhabdus indica*
bSNK8.5_TH	*Xenorhabdus indica*
bMSN19.5_TH	*Xenorhabdus japonica*
bKSN9.1_TH	*Xenorhabdus stockiae*
bPBI8.3_TH	*Xenorhabdus stockiae*
bPSD2.5_TH	*Xenorhabdus stockiae*
bKKN10.1_TH	*Xenorhabdus thuongxuanensis*

2. Evaluation of symbiotic bacteria against *Aedes* larvae

Larvicidal activities bioassay of *Photorhabdus* and *Xenorhabdus* against *Aedes* larvae was followed from previous study with some modifications (Yooyangket et al., 2018). The adjusted concentration of bacterial suspension was exposed against the third and fourth instar larvae of *Ae. aegypti* and *Ae. albopictus*. Ten larvae of each *Aedes* species were placed on a 24-well plate (2 cm^2 surface area per well) in triplicate. Any excess water was removed by pipette. Two millilitres of the adjusted bacterial suspension were added to each well. *Escherichia coli* ATCC 25922 and sterile distilled water were used as a control. The plate was incubated at RT (25–28◦C) under 12L:12D conditions to derive larvicidal activity. The total number of dead larvae was counted after 24, 48, 72, and 96 h exposure. Each experiment was performed thrice on different days. The mortality rate was calculated by averaging the nine replicates. The larvae that have pupated during the test period was negated from the test. If the pupation rate among the control larvae exceeds 10% during the experiment, the test will be invalidated and subsequently repeated. The mortality rate (percentages) of larvae in the control and treatment groups (each bacterial isolate) was analysed statistically using STATA version 13 (Kaplan–Meier Estimate, $P < 0.05$).

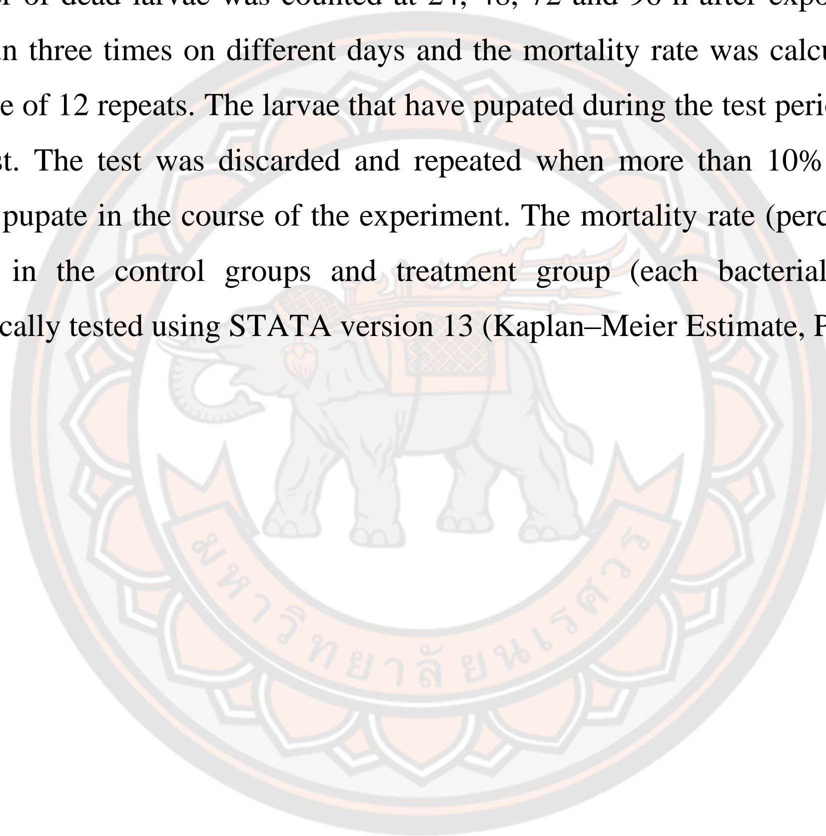
3. Larvicidal activities bioassay of bacterial extract against *Aedes* larvae

Two isolates of *Photorhabdus* and Two isolates of *Xenorhabdus* that show high mortality rate in previous larvicidal activities bioassay was sub-cultured on NBTA for crude compounds extraction. A single colony of each isolate was inoculated in a 2000-ml Erlenmeyer flask containing 500 ml LB. The flask was incubated in shaking incubator at 28°C, 180 rpm for 72 h. Then, a total 1000 ml ethyl acetate was added to the culture, mixed well and stood at RT for 24 h. All bacterial extracts were concentrated using a rotary vacuum evaporator (Buchi, Flawil, Switzerland) in triplicate. The extracts were weighted and stored at −20°C until using (Muangpat et al., 2017).

Larvicidal activities bioassay of the bacterial extract against *Aedes* larvae were performed according to the guideline of the World Health Organization (World Health Organization, 2005). The stored extracts were stood at RT for 30 min to return to normal temperature. After that, Dimethyl sulfoxide (DMSO) was added to dissolve

and adjusted the concentration of stock solution to 1%. The stock solution was diluted with DMSO in 10-fold dilution to 0.1, 0.01 and 0.001%.

Twenty-five *Aedes* larvae of each species were placed into 7-oz plastic cup containing 100 ml dechlorinate water (5 cm depth) in 4 replicate. Then, 1 ml of extract solution was added. Dechlorinated water and 2% DMSO were used as the negative control. The test of larvicidal activity against *Ae. aegypti* and *Ae. albopictus* was performed at 25–28ºC under 12L: 12D conditions. The total number of dead larvae was counted at 24, 48, 72 and 96 h after exposure. Each test was run three times on different days and the mortality rate was calculated from the average of 12 repeats. The larvae that have pupated during the test period was negated the test. The test was discarded and repeated when more than 10% of the control larvae pupate in the course of the experiment. The mortality rate (percentages) of the larvae in the control groups and treatment group (each bacterial isolate) were statistically tested using STATA version 13 (Kaplan–Meier Estimate, P<0.05).



CHAPTER IV

RESULTS

Prevalence of entomopathogenic nematodes in soil samples

A total of 1100 soil samples collected from 220 sites in 12 provinces of Thailand (Figure 17) yielded 118 EPNs (10.73%) belonging to 49 isolates of *Steinernema* (4.46%) and 69 isolates of *Heterorhabditis* (6.27%). Prevalence of EPNs in soil samples collected from 12 provinces of Thailand shows in Table 14. The highest number of EPNs was found in the Northeast of Thailand especially in Khon Kaen province. On the other hand, the EPNs were discovered in the smallest number in the North of Thailand. The greatest number of *Steinernema* and *Heterorhabditis* nematodes were obtained in the Northeast and Central region, respectively. The EPNs were recovered from loam (94.1%), clay loam (2.6%), gravelly soil (1.7%), sandy loam (0.8%), and clay (0.8%) with soil pH 4.4 - 7.0 (mean ± S.D. = 6.5 ± 0.6), temperature 23 - 32 ◦C (mean ± S.D. = 27.2 ± 1.8), and moisture 1.0 - 8.0 (mean ± S.D. = 2.0 ± 2.04). The soil samples without EPNs were not only recovered from loam, clay loam, gravelly soil, sandy loam and clay but also found in gravelly sandy loam and sand with soil pH 0.2 - 7.2 (mean ± S.D. = 6.3 ± 0.8), temperature 5 - 37 ◦C (mean ± S.D. = 27.3 ± 2.68), and moisture 0.0 - 8.5 (mean ± S.D. = 2.7 ± 2.39) (Table 15).

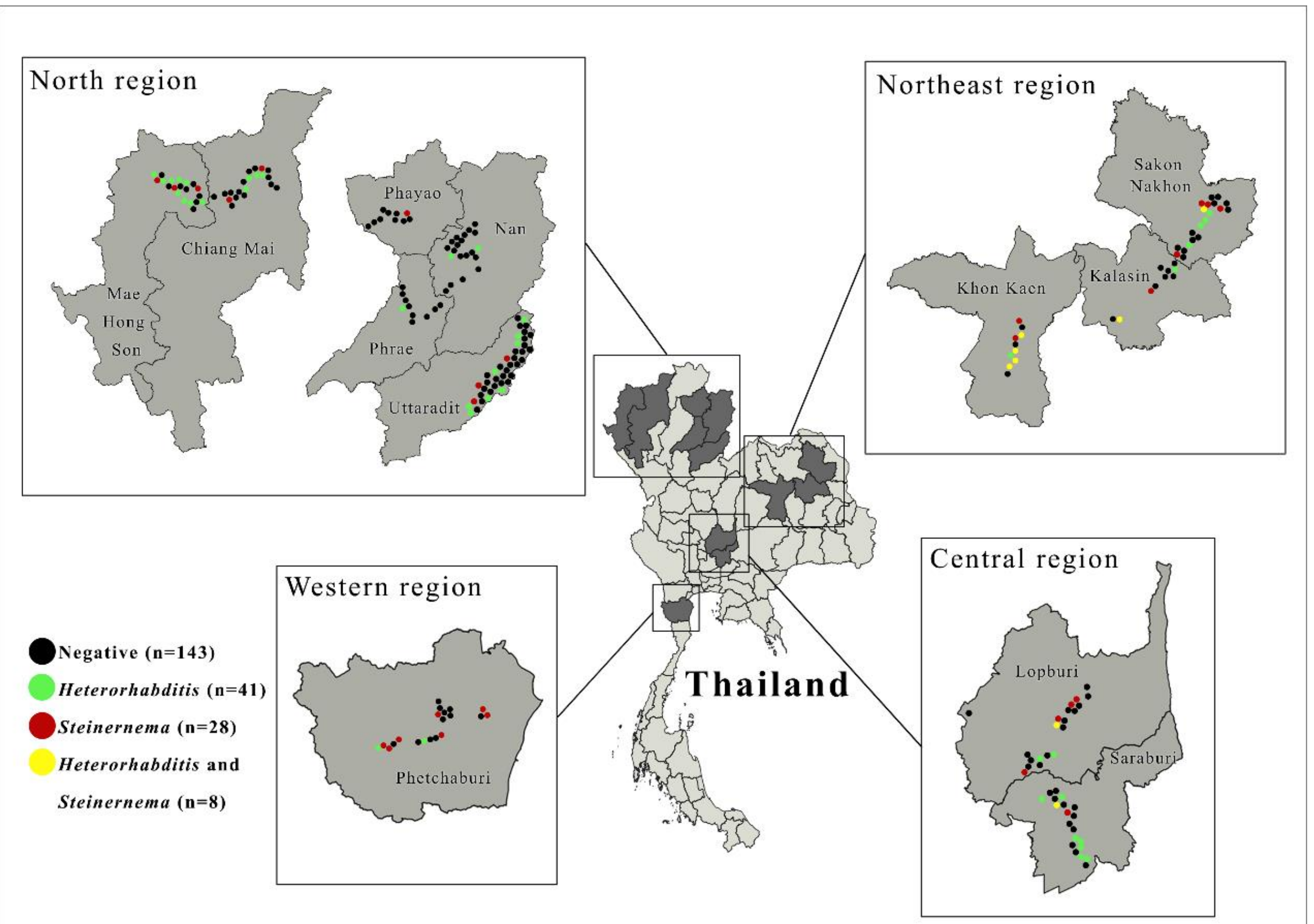


Figure 17 A map displaying soil sites in the 12 provinces covering four regions of Thailand

Table 14 Prevalence of entomopathogenic nematodes in soil samples collected from 12 provinces of Thailand

Region	Province (Code)	Number of soil site	Number of soil sample	Number of soil samples positive for EPNs (%)		
				Steinernema	***Heterorhabditis***	**Total**
Central		40	200	7 (3.5%)	19 (9.5%)	26 (13%)
	Lopburi (LBI)	20	100	5	5	10
	Saraburi (SRI)	20	100	2	14	16
North		120	600	10 (1.6%)	30 (5%)	40 (6.6%)
	Chiang Mai (CMI)	20	100	3	5	8
	Mae Hong Son (MSN)	20	100	3	13	16
	Nan (NAN)	20	100	0	3	3
	Phayao (PYO)	10	50	1	0	1
	Phrae (PRE)	10	50	0	1	1
	Uttaradit (PSD)	40	200	3	8	11
Northeast		40	200	24 (12%)	18 (9%)	42 (21%)
	Kalasin (KSN)	10	50	3	2	5
	Khon Kaen (KKN)	10	50	9	7	16
	Sakon Nakhon (SNK)	20	100	12	9	21

Region	Province (Code)	Number of soil site	Number of soil sample	Number of soil samples positive for EPNs (%)		
				Steinernema	*Heterorhabditis*	Total
West		20	100	8 (8%)	2 (2%)	10 (10%)
	Phetchaburi (PBI)	20	100	8	2	10
Total		220	1,100	49 (4.46%)	69 (6.27%)	118 (10.73%)

Table 15 Soil temperature, pH and moisture of the samples with EPNs and without EPNs (S.D. = Standard Deviation)

Soil parameters	Soil samples with EPNs (n=118)				Soil samples without EPNs (n=982)			
	Minimum value	Maximum value	Average	S.D.	Minimum value	Maximum value	Average	S.D.
Moisture	1.0	8.0	2.0	2.04	0.0	8.5	2.7	2.39
pH	4.4	7.0	6.5	0.60	0.2	7.2	6.3	0.80
Temperature (ºC)	23	32	27.2	1.80	5.0	37.0	27.3	2.68

Molecular identification of entomopathogenic nematodes

The soil samples were collected from 4 regions of Thailand including Central, North, Northeast and Western region which recovered 49 *Steinernema* isolates. According the analysis of the 28S rDNA (743 bp), 18 isolates were identified as *Steinernema surkhetense* with 99-100% BLASTN-similarity and one isolate was identified as *Steinernema kushidai* with 99% BLASTN-similarity. In addition, five *Steinernema* isolates were identified as *Steinernema* sp. YNd80, *Steinernema* sp. YNc215, *Steinernema guangdongense*, *Steinernema siamkayai*, and *Steinernema huense* (99% BLASTN-similarity for each isolate) based on 815 bp of the ITS region analysis. All the nucleotides of 24 *Steinernema* isolates were deposited in a GenBank database. The remaining 25 isolates were identified as genus *Steinernema*.

Heterorhabditis (69 isolates) was also recovered from the soil samples. Most EPNs (50 isolates) were identified as *Heterorhabditis indica* with a 99-100% similarity after BLASTN base on the analysis of the ITS region (722 bp). Four isolates of *Heterorhabditis* were identified as *Heterorhabditis* sp. SGmg3 with 98-99% identity, and three isolates were identified as *Heterorhabditis baujardi* with 99% similarity. The remaining 12 isolates were identified as genus *Heterorhabditis*.

All accession numbers of identified *Steinernema* and *Heterorhabditis* were shown in table 16.

Table 16 Accession number of identified *Steinernema* and *Heterorhabditis* nematodes

Accession number	Code	EPN maximum identity to
MZ457714	eCMI3.3_TH	*S. kushidai*
MZ457715	eKKN8.2_TH	*S. surkhetense*
MZ457716	eKSN9.1_TH	
MZ457717	eKSN9.4_TH	
MZ457718	eLBI3.4_TH	
MZ457719	eLBI14.5_TH	
MZ457720	eLBI15.3_TH	
MZ457721	ePBI2.4_TH	
MZ457722	ePBI3.2_TH	
MZ457723	ePBI8.3_TH	
MZ457724	ePBI10.4_TH	
MZ457725	ePBI11.1_TH	
MZ457726	ePBI19.5_TH	
MZ457727	ePBI20.3_TH	
MZ457728	ePSD2.5_TH	
MZ457729	ePYO8.5_TH	
MZ457730	eSNK9.3_TH	
MZ457731	eSRI6.3_TH	
MZ457732	eKKN10.3_TH	
MZ457735	eCMI14.1_TH	*Steinernema* sp. YNd80
MZ457736	eKKN5.5_TH	*S. siankayai*
MZ457737	eKKN10.1_TH	*S. guangdongense*
MZ457738	eKSN8.2_TH	*S. huense*
MZ457739	eSNK8.1_TH	*Steinernema* sp. YNc215
MZ474688	eCMI12.1_TH	*H. indica*
MZ474689	eCMI13.1_TH	
MZ474690	eCMI15.3_TH	
MZ474691	eKKN2.1_TH	
MZ474692	eKKN2.4_TH	

Accession number	Code	EPN maximum identity to
MZ474693	eKKN3.1_TH	*H. indica*
MZ474694	eKKN4.2_TH	
MZ474695	eKKN5.3_TH	
MZ474696	eKKN8.3_TH	
MZ474697	eKKN8.5_TH	
MZ474698	eKSN9.2_TH	
MZ474699	eLBI6.2_TH	
MZ474700	eLBI7.3_TH	
MZ474701	eLBI9.2_TH	
MZ474702	eLBI9.4_TH	
MZ474703	eLBI9.5_TH	
MZ474704	eMSN1.2_TH	
MZ474705	eMSN5.2_TH	
MZ474706	eMSN5.3_TH	
MZ474707	eMSN8.4_TH	
MZ474708	eMSN15.1_TH	
MZ474709	eMSN15.2_TH	
MZ474710	eMSN16.4_TH	
MZ474711	eMSN20.4_TH	
MZ474712	eMSN20.5_TH	
MZ474713	eNAN7.1_TH	
MZ474714	eNAN7.2_TH	
MZ474715	eNAN12.5_TH	
MZ474716	ePBI1.3_TH	
MZ474717	ePBI6.2_TH	
MZ474718	ePRE6.3_TH	
MZ474719	ePSD6.2_TH	
MZ474720	ePSD10.2_TH	
MZ474721	ePSD18.2_TH	
MZ474722	ePSD28.2_TH	
MZ474723	eSNK9.1_TH	
MZ474724	eSNK10.1_TH	
MZ474725	eSNK11.4_TH	

Accession number	Code	EPN maximum identity to
MZ474726	eSNK12.3_TH	*H. indica*
MZ474727	eSNK16.5_TH	
MZ474728	eSRI6.1_TH	
MZ474729	eSRI10.1_TH	
MZ474730	eSRI12.4_TH	
MZ474731	eSRI13.1_TH	
MZ474732	eSRI13.3_TH	
MZ474733	eSRI14.1_TH	
MZ474734	eSRI14.2_TH	
MZ474735	eSRI14.5_TH	
MZ474736	eSRI16.4_TH	
MZ474737	eSRI19.1_TH	
MZ474738	eMSN4.3_TH	*Heterorhabditis* sp. SGmg3
MZ474739	eMSN17.4_TH	
MZ474740	ePSD39.4_TH	
MZ474741	eSRI19.5_TH	
MZ474742	eSNK16.1_TH	*H. baujardi*
MZ474743	eSNK16.2_TH	
MZ474744	eSNK16.3_TH	

Isolation and identification of symbiotic bacteria

A total of 118 symbiotic bacteria were isolated from the EPNs and identified as *Photorhabdus* (69 isolates) and *Xenorhabdus* (49 isolates) based on the colony morphology. Nucleotide sequencing and a BLASTN search using 648 nucleotides from a partial sequence in the *recA* gene indicated that the majority of the symbiotic bacteria isolates (41 isolates) were *Photorhabdus luminescens* subsp. *akhurstii* with 97–100% similarity. Thirteen *Photorhabdus* isolates were identified as *Photorhabdus luminescens* subsp. *hainanensis* with 98–100% similarity, and six isolates were identified as *Photorhabdus asymbiotica* subsp. *australis* with 99% similarity.

Analysis of the partial *recA* gene sequence (647 bp) indicated 22 *Xenorhabdus stockiae* isolates, 13 *Xenorhabdus indica* isolates, 4 *Xenorhabdus griffiniae* isolates, 2 *Xenorhabdus japonica* isolates and 2 *Xenorhabdus thuongxuanensis* isolates. BLASTN similarity was markedly high at 97-100%. One isolate was identified as *Xenorhabdus eapokensis* by the 16S rRNA nucleotide sequence (1408 bp) with 100 % similarity. The remaining nine *Photorhabdus* isolates and five *Xenorhabdus* isolates were identified at the genus level. The nucleotide sequences of identified *Photorhabdus* and *Xenorhabdus* bacteria were submitted in a GenBank. Accession numbers were shown in table 17.

Table 17 Accession number of identified *Photorhabdus* and *Xenorhabdus* bacteria

Accession number	Code	EPN maximum identity to
ON751566	bCMI10.2_TH	*P. luminescens* subsp. *akhurstii*
ON751567	bCMI12.1_TH	
ON751568	bCMI13.1_TH	
ON751569	bCMI15.3_TH	
ON751570	bCMI15.4_TH	
ON751571	bKKN3.1_TH	
ON751572	bKKN4.2_TH	
ON751573	bKSN3.1_TH	
ON751574	bKSN9.2_TH	
ON751575	bLBI9.4_TH	
ON751576	bMSN1.2_TH	
ON751577	bMSN5.3_TH	
ON751578	bMSN10.3_TH	
ON751579	bMSN15.1_TH	
ON751580	bMSN15.2_TH	
ON751581	bMSN16.4_TH	
ON751582	bMSN17.1_TH	
ON751583	bMSN17.4_TH	
ON751584	bMSN20.5_TH	

Accession number	Code	EPN maximum identity to
ON751585	bNAN7.1_TH	*P. luminescens* subsp. *akhurstii*
ON751586	bNAN12.5_TH	
ON751587	bPSD10.2_TH	
ON751588	bPSD17.3_TH	
ON751589	bPSD27.3_TH	
ON751590	bPSD28.2_TH	
ON751591	bPSD39.4_TH	
ON751592	bPSD40.1_TH	
ON751593	bSNK11.4_TH	
ON751594	bSNK12.3_TH	
ON751595	bSNK16.1_TH	
ON751596	bSNK16.2_TH	
ON751597	bSNK16.3_TH	
ON751598	bSNK16.4_TH	
ON751599	bSNK16.5_TH	
ON751600	bSRI13.1_TH	
ON751601	bSRI14.1_TH	
ON751602	bSRI14.2_TH	
ON751603	bSRI14.5_TH	
ON751604	bSRI16.4_TH	
ON751605	bSRI19.1_TH	
ON751606	bSRI19.5_TH	
ON751607	bKKN2.1_TH	*P. luminescens* subsp. *hainanensis*
ON751608	bKKN2.4_TH	
ON751609	bKKN8.3_TH	
ON751610	bKKN8.5_TH	
ON751611	bLBI6.2_TH	
ON751612	bLBI9.2_TH	
ON751613	bLBI9.5_TH	
ON751614	bMSN8.4_TH	
ON751615	bPBI1.3_TH	
ON751616	bPBI6.2_TH	
ON751617	bPRE6.3_TH	

Accession number	Code	EPN maximum identity to
ON751618	bSNK10.1_TH	*P. luminescens* subsp. *akhurstii*
ON751619	bSRI10.2_TH	
ON751620	bMSN4.3_TH	*P. luminescens* subsp. *hainanensis*
ON751621	bSNK9.1_TH	
ON751622	bSRI6.1_TH	
ON751623	bSRI10.1_TH	
ON751624	bSRI10.3_TH	
ON751625	bSRI18.3_TH	
ON751691	bCMI14.4_TH	*X. stockiae*
ON751692	bKKN1.5_TH	
ON751693	bKKN4.1_TH	
ON751694	bKKN8.2_TH	
ON751695	bKSN8.2_TH	
ON751696	bKSN9.1_TH	*X. indica*
ON751697	bKSN9.4_TH	
ON751698	bLBI3.4_TH	
ON751699	bLBI11.5_TH	
ON751700	bLBI14.5_TH	
ON751701	bLBI15.3_TH	
ON751702	bPBI8.3_TH	
ON751703	bPBI10.2_TH	
ON751704	bPBI10.4_TH	
ON751705	bPBI19.5_TH	
ON751706	bPBI20.3_TH	
ON751707	bPSD2.5_TH	
ON751708	bPSD15.2_TH	
ON751709	bPSD34.2_TH	
ON751710	bSNK9.3_TH	
ON751711	bSRI3.5_TH	
ON751712	bSRI6.3_TH	
ON751713	bKKN10.3_TH	
ON751714	bKKN10.4_TH	
ON751715	bKKN10.5_TH	

Accession number	Code	EPN maximum identity to
ON751716	bSNK3.5_TH	*X. indica*
ON751717	bSNK7.2_TH	
ON751718	bSNK7.3_TH	
ON751719	bSNK7.4_TH	
ON751720	bSNK8.1_TH	
ON751721	bSNK8.5_TH	
ON751722	bSNK20.1_TH	
ON751723	bSNK20.2_TH	
ON751724	bSNK20.3_TH	
ON751725	bSNK20.4_T	
ON751726	bLBI9.1_TH	*X. griffiniae*
ON751727	bMSN3.3_TH	
ON751728	bMSN11.4_TH	
ON751729	bSNK9.4_TH	
ON751730	bCMI3.3_TH	*X. japonica*
ON751731	bMSN19.5_T	
ON751732	bKKN5.5_TH	*X. thuongxuanensis*
ON751733	bKKN10.1_T	
OM832407	bKKN2.5_TH	*X. eapokensis*

Phylogeny of *Steinernema* and *Heterorhabditis* nematodes

Phylogenetic trees were generated base on the neighbor-joining (NJ), maximum likelihood (ML), and Bayesian Inference (BI) methods. The phylogenetic tree of the 28S rDNA region of *Steinernema* found that 18 isolates were clustered with *S. surkhetense* (accession no. MF621005), whereas only one isolate was clustered with *S. kushidai* (accession no. AF331897) (Figure 18). The ITS region phylogenetic tree showed five groups of each isolate including *S. siamkayai* (accession no. JN571085), *S. huense* (accession no. KF857581), *Steinernema* sp. YNd80 (accession no. GU395632), *Steinernema* sp. YNc215 (accession no. GU395619), and *S. guangdongense* (accession no. AY170341) (Figure 19). Furthermore, the phylogenetic tree of the ITS region of *Heterorhabditis* showed 50 isolates clustered with *H. indica* (accession no. KF247222), 3 isolates clustered with

H. baujardi (accession no. AF548768), and 4 isolates clustered with *Heterorhabditis* sp. SGmg3 (accession no. FJ751864) (Figure 20).

Phylogeny of *Photorhabdus* and *Xenorhabdus* bacteria

Phylogenetic trees were generated base on the NJ, ML, and BI methods. The partial *recA* gene phylogenetic tree of *Photorhabdus* revealed thirteen isolates were grouped with *P. luminescens* subsp. *hainanensis* (accession no. FJ862004), fourty one isolates were grouped with *P. luminescens* subsp. *akhurstii* (accession no. FJ862005), and six isolates were grouped with *P. asymbiotica* subsp. *australis* (accession no. FJ862018) (Figure 21). Moreover, the partial *recA* gene phylogenetic tree of *Xenorhabdus* showed twenty isolates grouped with *X. stockiae* (accession no. JX485977), thirteen isolates grouped with *X. indica* (accession no. FJ823421), two isolates grouped with *X. japonica* (accession no. FJ823400), four isolates grouped with *X. griffiniae* (accession no. FJ823399) and two isolates grouped with *X. thuongxuanensis* (accession no. KX602194) (Figure 22). The only one *Xenorhabdus* isolate that constructed phylogenetic tree with partial 16s rRNA was grouped with *X. eapokensis* (accession no. NR156925) (Figure 23).

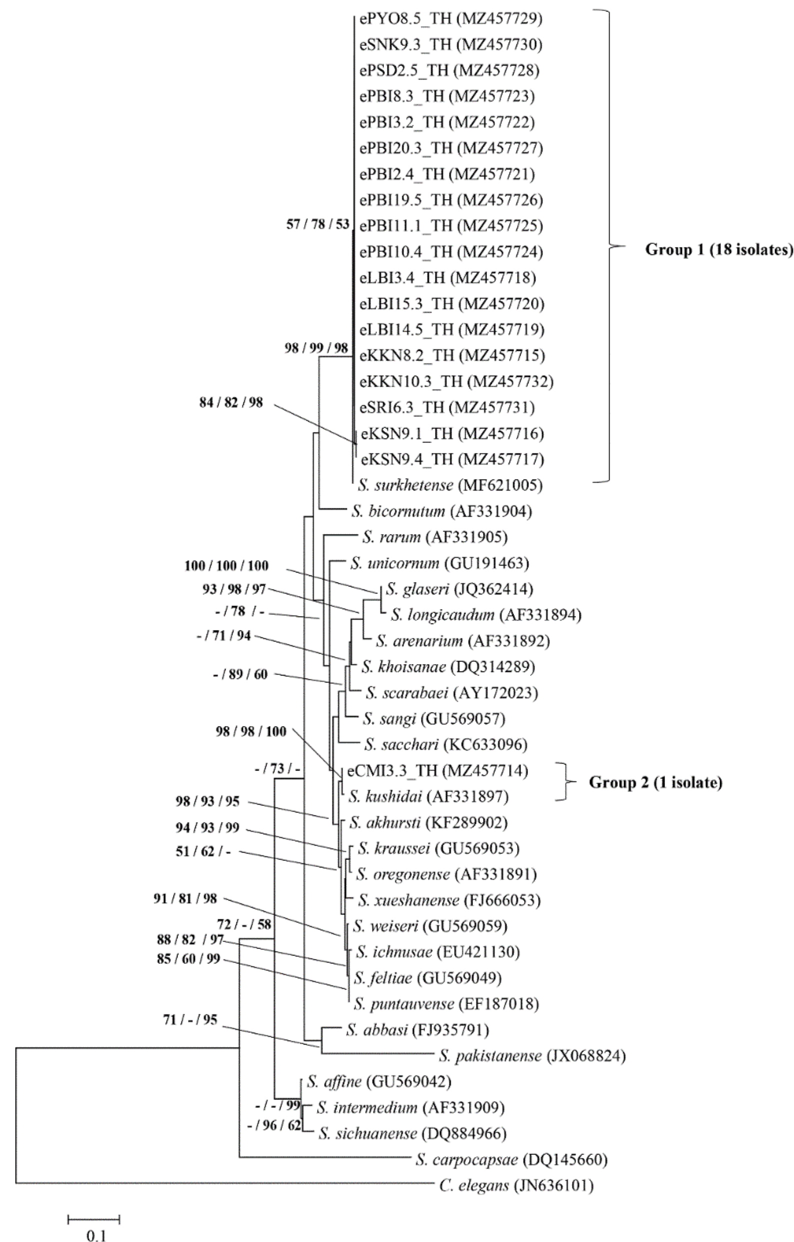


Figure 18 The maximum likelihood phylogenetic tree of *Steinernema* nematode constructed using the partial 28S rDNA region (743 bp) for 19 *Steinernema* isolates from Thailand and 26 *Steinernema* sequences of the 28S rDNA partial region obtained from NCBI (Tamura-Nei-parameter, 1000 bootstrap). *C. elegans* represented as the outgroup. Support values were shown on the branches; neighbor-joining bootstrap, maximum likelihood bootstrap and Bayesian posterior probabilities

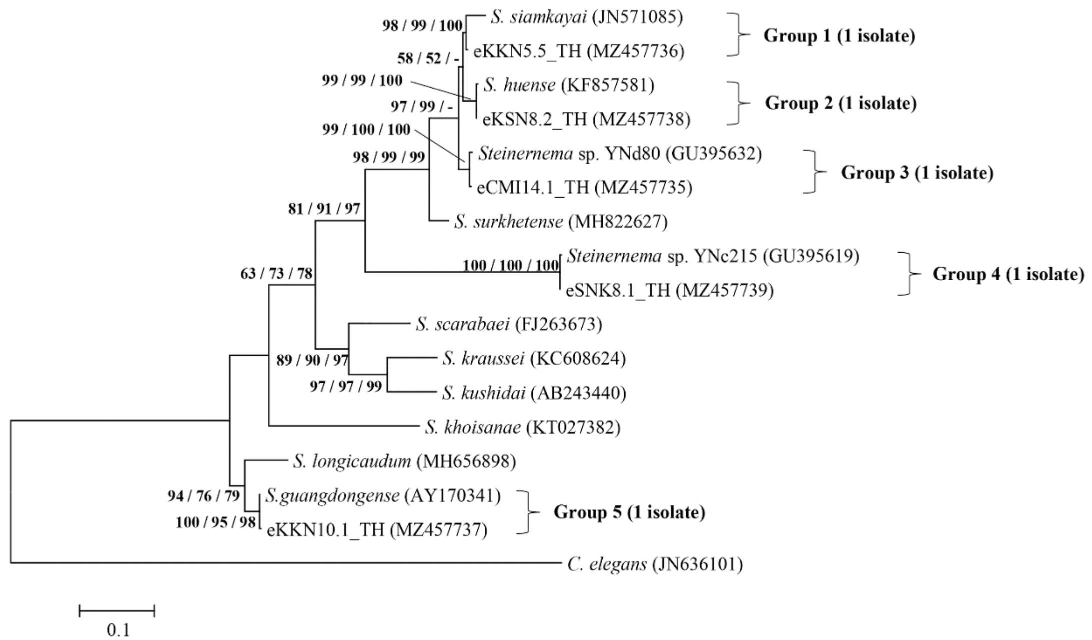


Figure 19 The maximum likelihood phylogenetic tree of *Steinernema* nematode constructed using the partial ITS region (815 bp) for 5 *Steinernema* isolates from Thailand and 11 *Steinernema* sequences of the ITS partial region obtained from NCBI (Tamura-Nei-parameter, 1000 bootstrap). *Caenorhabditis elegans* represented as the outgroup. Support values were shown on the branches; neighbor-joining bootstrap, maximum likelihood bootstrap and Bayesian posterior probabilities

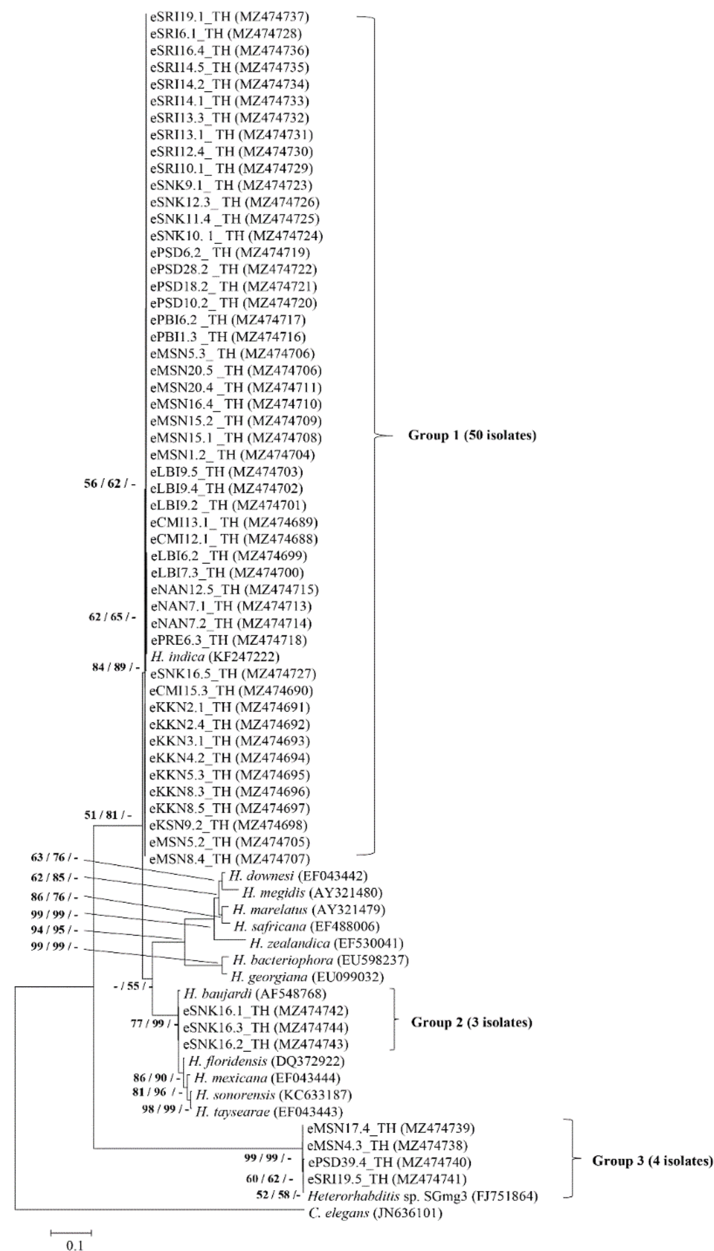


Figure 20 The maximum likelihood phylogenetic tree of *Heterorhabditis* nematode constructed using the partial ITS region (722 bp) for 57 *Heterorhabditis* isolates from Thailand and 14 *Heterorhabditis* sequences of the ITS partial region obtained from NCBI (Tamura-Nei-parameter, 1000 bootstrap). *Caenorhabditis elegans* represented as the outgroup. Support values were shown on the branches; neighbor-joining bootstrap, maximum likelihood bootstrap and Bayesian posterior probabilities

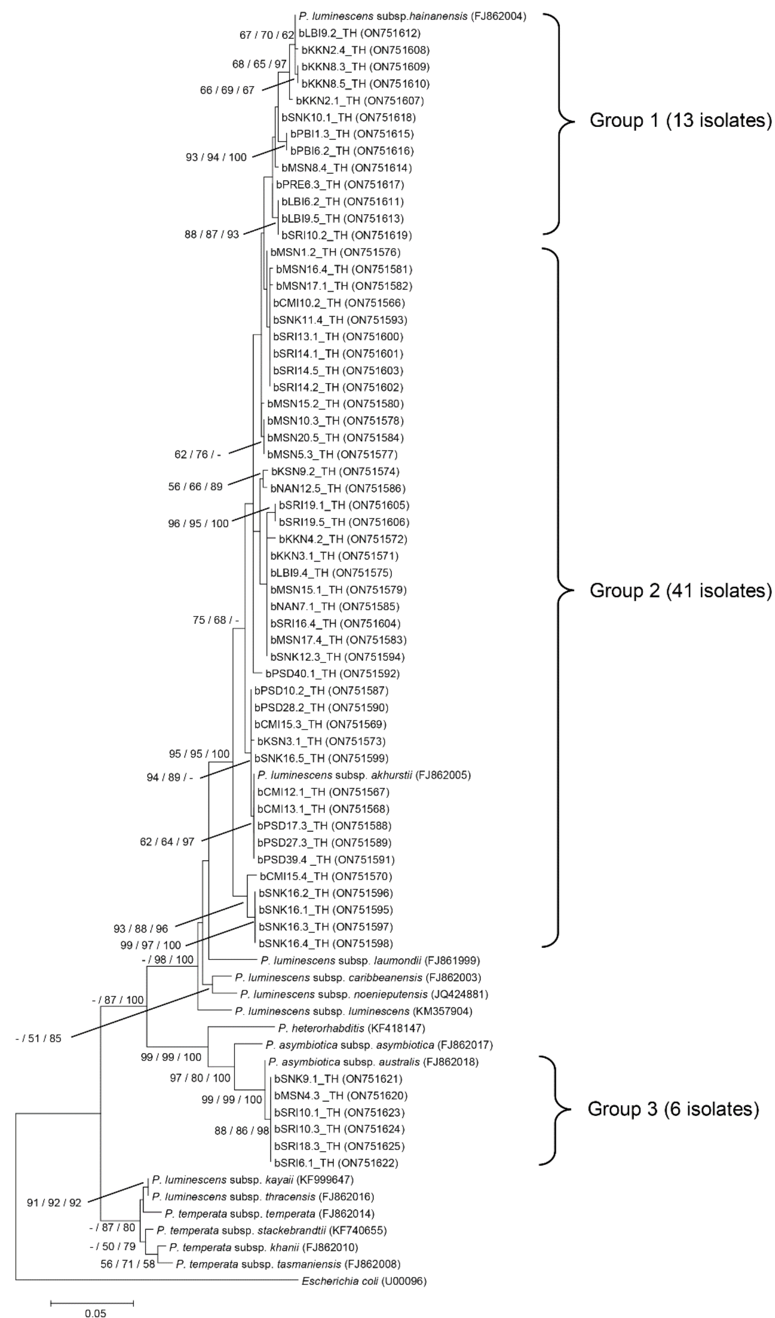


Figure 21 The maximum likelihood phylogenetic tree of *Photorhabdus* bacteria constructed using the partial *recA* gene (648 bp) for 60 *Photorhabdus* isolates from Thailand and 15 *Photorhabdus* sequences of the partial *recA* gene obtained from NCBI (Tamura-Nei-parameter, 1000 bootstrap). *Escherichia coli* (*E. coli*) represented as the outgroup. Support values were shown on the branches; neighbor-joining bootstrap, maximum likelihood bootstrap and Bayesian posterior probabilities

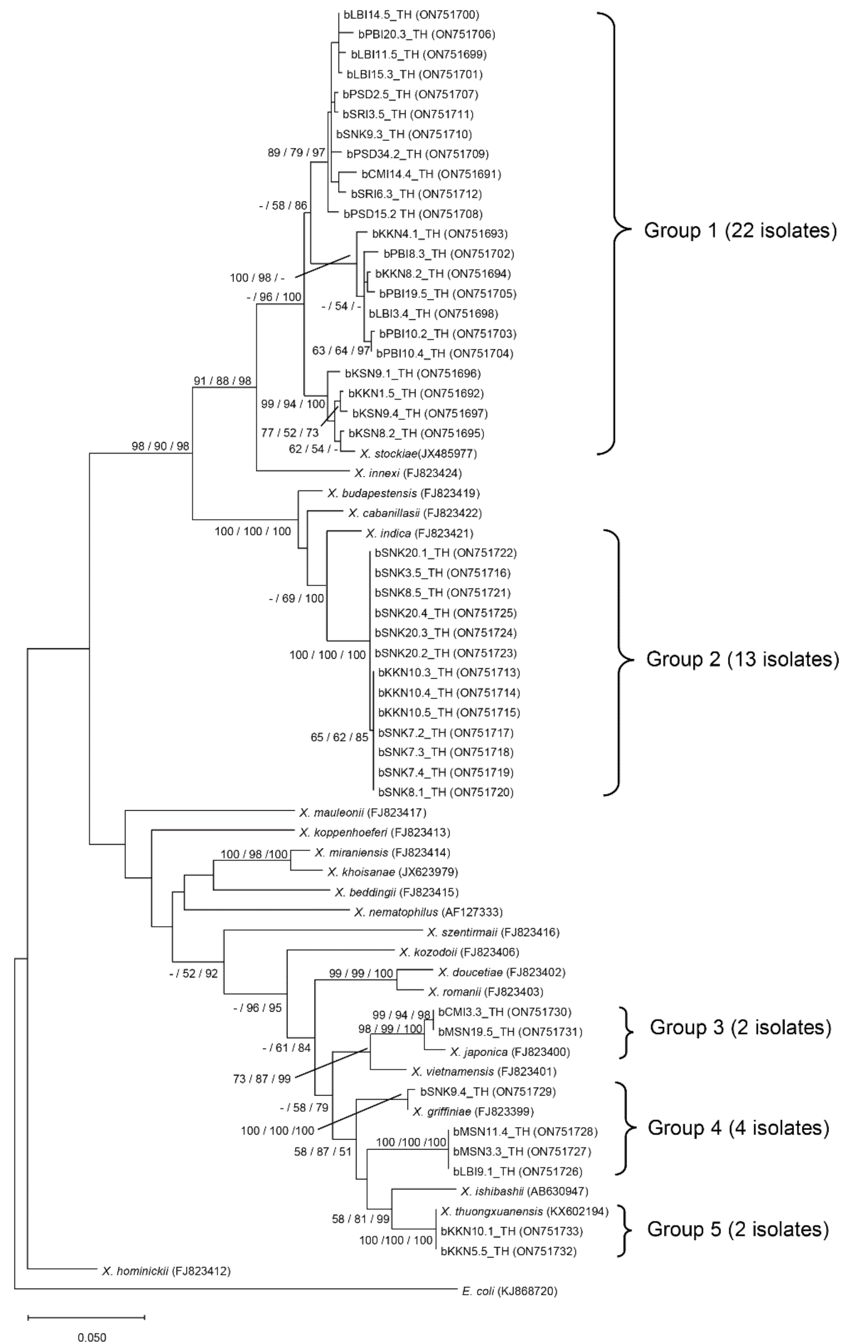


Figure 22 The maximum likelihood phylogenetic tree of *Xenorhabdus* bacteria constructed using the partial *recA* gene (647 bp) for 43 *Xenorhabdus* isolates from Thailand and 21 *Xenorhabdus* sequences of the partial *recA* gene obtained from NCBI (Tamura-Nei-parameter, 1000 bootstrap). *E. coli* represented as the outgroup. Support values were shown on the branches; neighbor-joining bootstrap, maximum likelihood bootstrap and Bayesian posterior probabilities

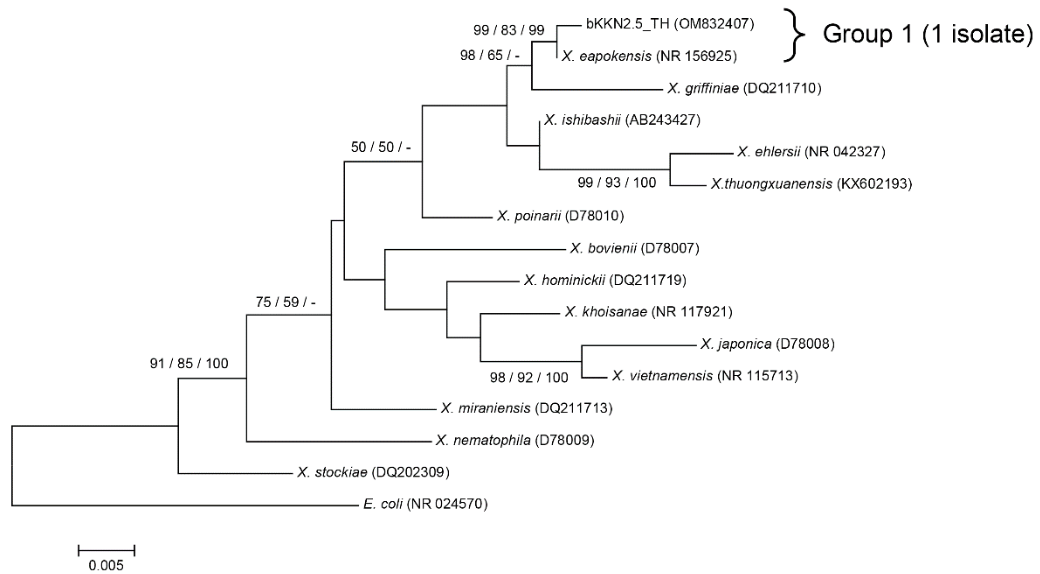


Figure 23 The maximum likelihood phylogenetic tree of *Xenorhabdus* bacteria constructed using the partial 16S rRNA gene (1408 bp) for a *Xenorhabdus* isolate from Thailand and 15 *Xenorhabdus* sequences of the partial 16S rRNA gene obtained from NCBI (Tamura-Nei-parameter, 1000 bootstrap). *E. coli* represented as the outgroup. Support values were shown on the branches; neighbor-joining bootstrap, maximum likelihood bootstrap and Bayesian posterior probabilities

Association between symbiotic bacteria and EPN hosts

The symbiotic associations of *Photorhabdus* with *Heterorhabditis* and *Xenorhabdus* with *Steinernema* are shown in Table 18. Most *P. luminescens* subsp. *akhurstii* isolates were associated with *H. indica* and most *X. stockiae* isolates were associated with *S. surkhetense*. In addition, *P. luminescens* subsp. *akhurstii* was associated with *H. baujardi*, *Heterorhabditis* sp. SGmg3., and an unidentified *Heterorhabditis* spp. *Photorhabdus asymbiotica* subsp. *australis* was found to have a symbiotic relationship with *H. indica*, *Heterorhabditis* sp. SGmg3. and an unidentified *Heterorhabditis* spp. *Photorhabdus luminescens* subsp. *hainanensis* was symbiotic with *H. indica* and an unidentified *Heterorhabditis* spp. *Photorhabdus* spp. was associated with *H. indica*. Some *X. stockiae* isolates were also associated with *S. huense* and an unidentified *Steinernema*. *Xenorhabdus indica* had a symbiotic relationship with *S. surkhetense*, *Steinernema* spp. YNc215 and an unidentified *Steinernema*. *Xenorhabdus thuongxuanensis* was associated with *S. guangdongense* and *S. siamkayai*. *Xenorhabdus japonica* was associated with *S. kushidai* and an unidentified *Steinernema*. *Xenorhabdus eapokensis*, and *X. griffiniae* were associated with an unidentified *Steinernema*. *Xenorhabdus* spp. was also symbiotic with *S. surkhetense* and *Steinernema* spp. YNd80.

Table 18 Association between symbiotic bacteria and EPNs in the 12 Provinces of Thailand

Entomopathogenic nematode	Symbiotic bacteria	Code
Heterorhabditis baujardi	*Photorhabdus luminescens* subsp. *akhurstii*	bSNK16.1_TH, bSNK16.2_TH, bSNK16.3_TH
Heterorhabditis indica	*Photorhabdus asymbiotica* subsp. *australis*	bSNK9.1_TH, bSRI6.1_TH, bSRI10.1_TH

Entomopathogenic nematode	Symbiotic bacteria	Code
Heterorhabditis indica	*Photorhabdus luminescens* subsp. *hainanensis*	bKKN2.1_TH, bKKN2.4_TH, bKKN8.3_TH, bKKN8.5_TH, bLBI6.2_TH, bLBI9.2_TH, bLBI9.5_TH, bMSN8.4_TH, bPBI1.3_TH, bPBI6.2_TH, bPRE6.3_TH, bSNK10.1_TH
	Photorhabdus luminescens subsp. *akhurstii*	bCMI12.1_TH, bCMI13.1_TH, bCMI15.3_TH, bKKN3.1_TH, bKKN4.2_TH, bKSN9.2_TH, bLBI9.4_TH, bMSN1.2_TH, bMSN5.3_TH, bMSN15.1_TH, bMSN15.2_TH, bMSN16.4_TH, bMSN20.5_TH, bNAN7.1_TH, bNAN12.5_TH, bPSD10.2_TH, bPSD28.2_TH, bSNK11.4_TH, bSNK12.3_TH, bSNK16.5_TH,

Entomopathogenic nematode	Symbiotic bacteria	Code
Heterorhabditis indica	*Photorhabdus luminescens* subsp. *akhurstii*	bSRI13.1_TH, bSRI14.1_TH, bSRI14.2_TH, bSRI14.5_TH, bSRI16.4_TH, bSRI19.1_TH
	Photorhabdus spp.	bKKN5.3_TH, bLBI7.3_TH, bMSN5.2_TH, bMSN20.4_TH, bNAN7.2_TH, bPSD6.2_TH, bPSD18.2_TH, bSRI12.4_TH, bSRI13.3_TH
Heterorhabditis sp. SGmg3	*Photorhabdus asymbiotica* subsp. *australis*	bMSN4.3_TH
	Photorhabdus luminescens subsp. *akhurstii*	bPSD39.4_TH, bMSN17.4_TH, bSRI19.5_TH
Heterorhabditis spp.	*Photorhabdus asymbiotica* subsp. *australis*	bSRI10.3_TH, bSRI18.3_TH
	Photorhabdus luminescens subsp. *hainanensis*	bSRI10.2_TH
	Photorhabdus luminescens subsp. *akhurstii*	bCMI10.2_TH, bCMI15.4_TH, bKSN3.1_TH, bMSN10.3_TH, bMSN17.1_TH, bPSD17.3_TH, bPSD27.3_TH,

Entomopathogenic nematode	Symbiotic bacteria	Code
Heterorhabditis spp.	*Photorhabdus luminescens* subsp. *akhurstii*	bPSD40.1_TH, bSNK16.4_TH
Steinernema guangdongense	*Xenorhabdus thuongxuanensis*	bKKN10.1_TH
Steinernema huense	*Xenorhabdus stockiae*	bKSN8.2_TH
Steinernema kushidai	*Xenorhabdus japonica*	bCMI3.3_TH
Steinernema siamkayai	*Xenorhabdus thuongxuanensis*	bKKN5.5_TH
Steinernema surkhetense	*Xenorhabdus indica*	bPSD2.5_TH
	Xenorhabdus stockiae	bKKN8.2_TH, bKSN9.1_TH, bKSN9.4_TH, bLBI3.4_TH, bLBI14.5_TH, bLBI15.3_TH, bPBI8.3_TH, bPBI10.4_TH, bPBI11.1_TH, bPBI19.5_TH, bPBI20.3_TH, bSNK9.3_TH, bSRI6.3_TH
	Xenorhabdus spp.	bKKN10.3_TH, bPBI2.4_TH, bPBI3.2_TH, bPYO8.5_TH
Steinernema sp. YNc215	*Xenorhabdus indica*	bSNK8.1_TH
Steinernema sp. YNd80	*Xenorhabdus* spp.	bCMI14.1_TH
Steinernema spp.	*Xenorhabdus eapokensis*	bKKN2.5_TH
	Xenorhabdus griffinae	bLBI9.1_TH, bMSN11.4_TH, bMSN3.3_TH, bSNK9.4_TH

Entomopathogenic nematode	Symbiotic bacteria	Code
Steinernema spp.	*Xenorhabdus indica*	bSNK3.5_TH, bSNK7.2_TH, bSNK7.3_TH, bSNK7.4_TH, bSNK8.5_TH, bKKN10.4_TH, bKKN10.5_TH, bSNK20.1_TH, bSNK20.2_TH, bSNK20.3_TH, bSNK20.4_TH
	Xenorhabdus japonica	bMSN19.5_TH
	Xenorhabdus stockiae	bCMI14.4_TH, bKKN1.5_TH, bKKN4.1_TH, bLBI11.5_TH, bPBI10.2_TH, bPSD15.2_TH, bPSD34.2_TH, bSRI3.5_TH

Efficacy of entomopathogenic nematodes against *Aedes aegypti* larvae

The *Steinernema surkhetense* isolate ePYO8.5_TH displayed significant virulence towards *Ae. aegypti* larvae in comparison to other EPN isolates and control group (dechlorinated water). In a 24-well plate, the mortality rate at 96 h post-exposure was 73 ± 20.60, 92 ± 9.37, 90 ± 10.44, and 92 ± 9.37% for treatment with 800, 1600, 3200, and 6400 IJs, respectively ($P < 0.0001$; df = 4). Conversely, all the isolates of *H. indica* (eMSN1.2_TH, eMSN4.3_TH, ePRE6.3_TH, and eLBI9.2_TH) exhibited low efficacy against *Ae. aegypti* larvae, with variable mortality rates ranging from 0 and 18% after 96 h exposure (Table 19 and Figure 24). However, the larvae showed significantly increased mortality after treatment with 1600, 3200 and 6400 IJs

of ePRE6.3_TH compared with control group (*P* < 0.0001; df = 4). In a six-well plate, *S. surkhetense* (ePYO8.5_TH) also demonstrated significant pathogenicity towards *Ae. aegypti* larvae as well. All concentrations of ePYO8.5_TH exhibited significant larval mortality from the first 24 h of treatment (*P* < 0.001; df = 4). The highest mortality rate at 96 h post-exposure was 78 ± 14.88, 75 ± 17.73, 89 ± 9.91, and 76 ± 19.23% for treatment with 4000, 8000, 16,000, and 32,000 IJs respectively (Table 20 and Figure 25). The EPNs were found throughout the entire body of the *Ae. aegypti* larvae, including head, thorax, abdomen, and hemocoel. Some live and mobile IJs of EPN were discovered within the body cavity of the mosquito larvae, along with black remains of EPNs in the haemocoel (Figure 26).

Table 19 Mortality rates of *Aedes aegypti* larvae after exposure to entomopathogenic nematode in 24-well plate (S.D. = Standard Deviation)

Entopathogenic nematode (Code)	No. of IJs	Mortality rate (%) ± S.D.			
		24 h	48 h	72 h	96 h
Steinernema surkenthense (ePYO8.5_TH)	800	68 ± 24.5	70 ± 23.74	70 ± 23.74	73 ± 20.60
	1600	85 ± 11.68	91 ± 9.00	92 ± 9.37	92 ± 9.37
	3200	85 ± 10.84	89 ± 10.84	89 ± 10.84	90 ± 10.44
	6400	82 ± 16.42	88 ± 15.86	88 ± 15.86	92 ± 9.37
Heterorhabditis indica (eMSN1.2_TH)	800	1 ± 2.89	1 ± 2.89	1 ± 2.89	1 ± 2.89
	1600	0 ± 0.00	0 ± 0.00	0 ± 0.00	1 ± 2.89
	3200	0 ± 0.00	0 ± 0.00	1 ± 2.89	1 ± 2.89
	6400	0 ± 0.00	0 ± 0.00	3 ± 4.52	3 ± 4.52
Heterorhabditis indica (eMSN4.3_TH)	800	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	1600	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
	3200	0 ± 0.00	0 ± 0.00	1 ± 2.89	1 ± 2.89
	6400	0 ± 0.00	0 ± 0.00	0 ±0.00	0 ± 0.00

Entopathogenic nematode (Code)	No. of IJs	Mortality rate (%) ± S.D.			
		24 h	48 h	72 h	96 h
Heterorhabditis indica (ePRE6.3_TH)	800	0 ± 0.00	0 ± 0.00	2 ± 3.89	3 ± 4.92
	1600	1 ± 2.89	3 ± 6.22	9 ± 10.84	13 ± 15.45
	3200	5 ± 11.68	7 ± 11.55	11 ± 11.65	13 ± 14.22
	6400	0 ± 0.00	8 ± 9.65	16 ± 15.64	18 ± 16.42
Heterorhabditis indica (eLBI9.2_TH)	800	1 ± 2.89	1 ± 2.89	1 ± 2.89	1 ± 2.89
	1600	0 ± 0.00	0 ± 0.00	0 ± 0.00	3 ± 8.66
	3200	0 ± 0.00	12 ± 16.97	13 ± 17.23	14 ± 16.76
	6400	0 ± 0.00	3 ± 4.52	3 ± 4.52	3 ± 4.52
Control; Dechlorinated water		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

Table 20 Mortality rates of *Aedes aegypti* larvae after exposure to *Steinernema surkenthense* (ePYO8.5_TH) in 6-well plate (S.D. = Standard Deviation)

Entopathogenic nematode (Code)	No. of IJs	Mortality rate (%) ± S.D.			
		24 h	48 h	72 h	96 h
Steinernema surkenthense (ePYO8.5_TH)	4000	58 ± 21.21	73 ± 12.82	75 ± 16.04	78 ± 14.48
	8000	60 ± 26.19	71 ± 16.42	74 ± 15.98	75 ± 17.73
	16000	70 ± 16.90	83 ± 12.82	83 ± 12.82	89 ± 9.91
	32000	54 ± 10.61	68 ± 17.53	74 ± 18.47	76 ± 19.23
Control; Dechlorinated water		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

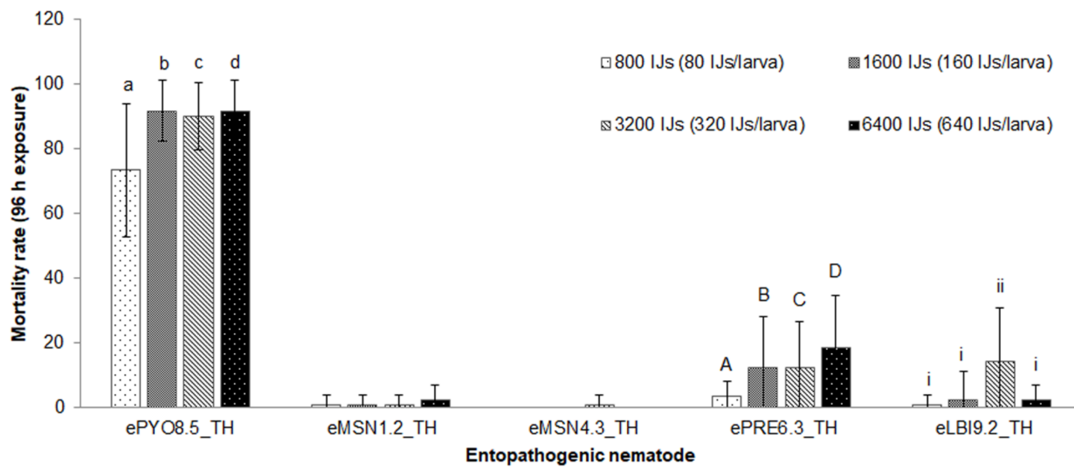


Figure 24 The mortality rate of the *Aedes aegypti* larvae against EPNs at 96 hours in a 24-well plate. The differences letters above each column were the significant differences between the concentrations of each EPN (P < 0.05). The mortality rate of control (dechlorinated water) was 0%

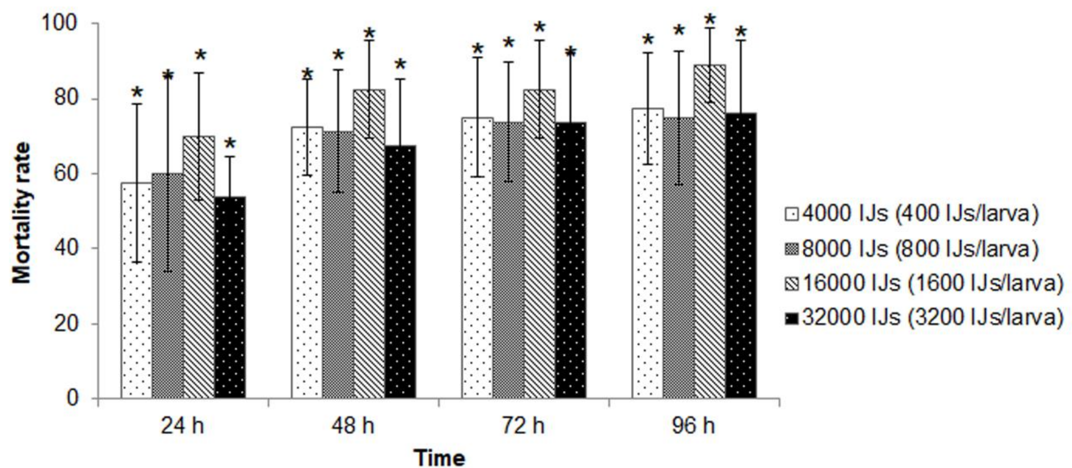


Figure 25 The mortality rate of the *Aedes aegypti* larvae against *Steinernema surkhetense* (ePYO8.5_TH) in a six-well plate. The asterisks above each column show the significant differences between the test group and the control group (*P* < 0.05). The mortality rate of control (dechlorinated water) was 0%

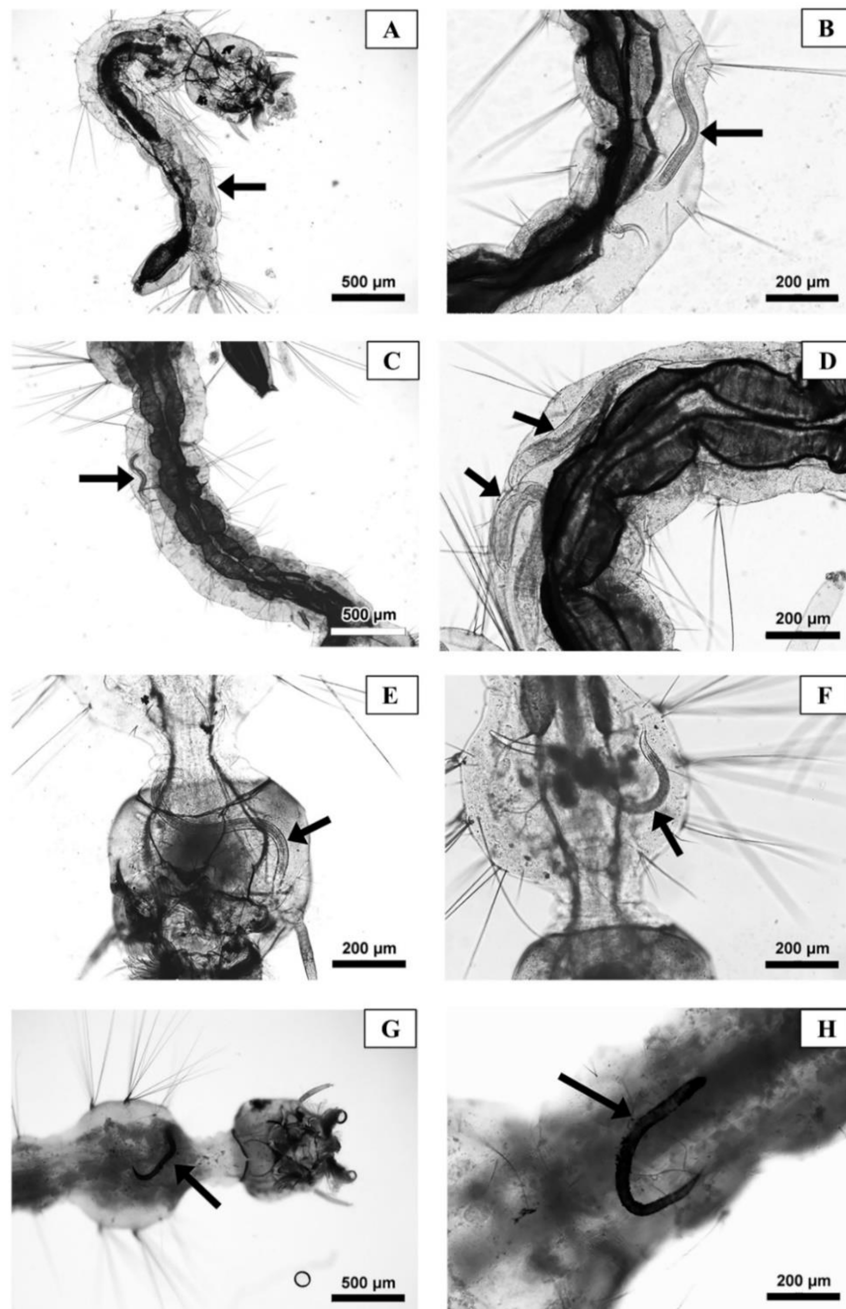


Figure 26 ***Aedes aegypti*** **larvae treated with EPNs.** ***Steinernema surkenthense*** **(ePYO8.3_TH) in the hemocoel of** ***Ae. aegypti*** **larva (A–D).** ***Heterorhabditis indica*** **(eLBI9.2_TH) in head (E), thorax (F and G), and body (H) of** ***Ae. aegypti*** **larva and its black remains (G and H). the black arrows indicate the EPNs**

Larvicidal activity of *Photorhabdus* and *Xenorhabdus* on *Aedes aegypti* and *Aedes albopictus*

The highest larvicidal activity of the symbiotic bacteria against *Ae. aegypti* larvae was observed in the *X. griffiniae* isolate bMSN3.3_TH, exhibiting a mortality rate of 90 ± 3.71 % after 96 hours of exposure (Table 21). A significant difference (Kaplan–Meier estimate, $P < 0.05$) was observed in the mortality rate of this isolate compared to the others. In contrast, the mortality rate for the control groups, consisting of *E. coli* ATCC 25922 and sterile distilled water remained at 0 % that demonstrated the significant difference between the experimental and control groups, except for *X. indica* isolate bSNK8.5_TH and *X. stockiae* isolate bKSN9.1_TH. While the other isolates displayed effectiveness against *Ae. aegypti* larvae, their efficacy was comparatively lower, resulting in mortality rates below 36%.

The outstanding larvicidal activity of the symbiotic bacteria against *Ae. albopictus* larvae unequivocally evidenced in the *X. griffiniae* isolate bMSN3.3_TH, yielding an 81 ± 2.13 % mortality rate. This mortality rate exhibited a consistent incremental trend from 48 to 96 hours subsequent to exposure (Table 21). A significant difference in the mortality rate (Kaplan–Meier estimate, P-value < 0.05) was observed between this and other isolates, except for *P. luminescens* subsp. *akhurstii* isolate bPSD40.1_TH. Additionally, the *X. indica* isolate bSNK8.5_TH showed high efficacy in controlling *Ae. albopictus* larvae, with a 76 ± 3.62 % mortality rate after 48 h exposure. A significant difference (Kaplan–Meier estimate, P-value < 0.05) was identified in the mortality rate between this and other isolates. Mortality rates of the control groups *E. coli* ATCC 25922 and sterile distilled water were 3 ± 0.28 % and 0 %, respectively. The mortality rates noted in the test groups and control groups were significantly different, except *P. asymbiotica* subsp. *australis* isolate bSRI10.1_TH. On the contrary, the other isolates showed low efficiency in controlling *Ae. albopictus* larvae (Figure 27).

Based on the ethyl acetate extract, the findings showed that only 1% of the extract solution could kill both types of *Aedes* larvae. The mortality rate in the control groups (2% DMSO and dechlorinated water) was 0%, with significant differences observed in the mortality rate (Kaplan-Meier estimate, P-value < 0.05) of the test and control groups. Notably, the 1 % *X. indica* crude extract solution isolate bSNK8.5_TH

showed the highest mortality rate in both *Ae. aegypti* and *Ae. albopictus* larvae 96 h after exposure, with 50 ± 3.66% and 35 ± 3.37%, respectively. A significant difference (Kaplan-Meier estimate, *P*-value < 0.05) in the mortality rate of the crude extract solutions and the other samples was observed, except for *X. griffiniae* isolate bMSN3.3_TH with *Ae. albopictus* (Figure 28 and Table 22).

Table 21 Mortality rates of *Aedes aegypti* and *Aedes albopictus* larvae after exposure to whole cell suspension of *Photorhabdus* and *Xenorhabdus* bacteria (S.D. was Standard Deviation)

Symbiotic bacteria	Isolate	Mortality rate (%) ± S.D.							
		Aedes aegypti				*Aedes albopictus*			
		24 h	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
Photorhabdus asymbiotica subsp. *australis*	bSRI10.1_TH	34 ± 3.50	36 ± 2.96	36 ± 2.54	36 ± 2.25	1 ± 0.33	2 ± 0.32	2 ± 0.27	2 ± 0.23
Photorhabdus luminescens subsp. *hainanensis*	bSRI10.2_TH	7 ± 0.71	23 ± 1.62	23 ± 1.42	23 ± 1.27	23 ± 2.12	44 ± 2.10	59 ± 1.97	60 ± 1.89
Photorhabdus luminescens subsp. *akhurstii*	bPSD40.1_TH	21 ± 2.42	28 ± 2.03	29 ±1.76	29 ± 1.58	36 ± 2.83	49 ± 2.62	62 ± 2.56	66 ± 2.36
Photorhabdus luminescens subsp. *akhurstii*	bCMI13.1_TH	1 ± 0.33	6 ± 0.96	6 ± 0.79	6 ± 0.68	21 ± 1.76	54 ± 2.52	56 ± 2.40	57 ± 2.21
Photorhabdus luminescens subsp. *akhurstii*	bSNK16.3_TH	10 ± 1.41	23 ± 2.01	24 ± 1.71	24 ± 1.52	11 ± 1.17	26 ± 1.32	29 ± 1.19	30 ± 1.11
Xenorhabdus eapokensis	bKKN2.5_TH	9 ± 2.67	9 ± 1.89	9 ± 1.54	9 ± 1.33	3 ± 1.00	9 ± 0.86	9 ± 0.72	9 ± 0.64
Xenorhabdus griffiniae	bMSN3.3_TH	21 ± 4.20	74 ± 4.39	89 ± 4.95	90 ± 3.71	18 ± 2.17	52 ± 2.43	64 ± 2.27	81 ±2.13
Xenorhabdus griffiniae	bLBI9.1_TH	0 ± 0.00	16 ± 2.37	16 ± 1.95	16 ± 1.69	39 ± 3.82	41 ± 3.26	42 ± 2.80	42 ± 2.50
Xenorhabdus indica	bKKN10.4_TH	28 ± 4.29	29 ± 3.26	29 ± 2.72	30 ± 2.38	4 ± 1.01	10 ± 0.99	10 ± 0.83	10 ± 0.73
Xenorhabdus indica	bSNK8.5_TH	1 ± 0.33	1 ± 0.42	1 ± 0.19	1 ± 0.17	71 ± 1.62	76 ± 3.62	76 ± 3.45	76 ± 3.35

Symbiotic bacteria	Isolate	Mortality rate (%) ± S.D.							
		Aedes aegypti				*Aedes albopictus*			
		24 h	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
Xenorhabdus japonica	bMSN19.5_TH	16 ± 1.81	16 ± 1.48	16 ± 1.25	16 ± 1.10	8 ± 1.64	12 ± 1.24	12 ± 1.05	12 ± 0.92
Xenorhabdus stockiae	bKSN9.1_TH	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	8 ± 1.09	11 ± 0.86	12 ± 0.75	12 ± 0.67
Xenorhabdus stockiae	bPBI8.3_TH	10 ± 3.00	10 ± 2.12	10 ± 1.73	10 ± 1.50	7 ± 1.12	9 ± 0.86	10 ± 0.73	10 ± 0.65
Xenorhabdus stockiae	bPSD2.5_TH	12 ± 2.28	27 ± 2.43	27 ± 2.07	27 ± 1.82	3 ± 0.71	6 ± 0.67	33 ± 2.59	33 ± 2.29
Xenorhabdus thuongxuanensis	bKKN10.1_TH	11 ± 3.33	32 ± 3.71	32 ± 3.10	32 ± 2.71	38 ± 4.71	38 ± 3.77	38 ± 3.18	38 ± 2.80
Escherichia coli ATCC 25922: negative control		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	2 ± 0.44	3 ± 0.38	3 ± 0.32	3 ± 0.28
Sterile distilled water: negative control		0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

Table 22 Mortality rates of *Aedes aegypti* and *Aedes albopictus* larvae after exposure to crude extract solution of *Photorhabdus* and *Xenorhabdus* (S.D. = Standard Deviation)

Symbiotic bacteria	isolate	Extract concen-tration (%)	Mortality rate (%) ± S.D.							
			Aedes aegypti				*Aedes albopictus*			
			24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
Photorhabdus asymbiotica subsp. *australis*	bSRI10.1_TH	1	1 ± 0.62	12 ± 2.15	23± 2.48	25 ± 2.24	3 ± 1.76	17 ± 2.18	23 ± 2.52	24 ± 2.29
		0.1	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.01	0 ± 0.00	0 ± 0.00	0 ± 0.00	1 ± 0.20	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.001	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Photorhabdus luminescens subsp. *akhurstii*	bPSD40.1_TH	1	0 ± 0.00	2 ± 0.55	4 ± 0.72	5 ± 0.65	2 ± 0.80	12 ± 2.70	22 ± 2.62	22 ± 2.39
		0.1	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.01	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.001	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Xenorhabdus griffiniae	bMSN3.3_TH	1	2 ± 1.00	8 ± 1.65	18 ± 2.18	20 ± 1.95	5 ± 2.93	23 ± 3.31	27 ± 2.91	28 ±2.67
		0.1	1 ± 0.62	1 ± 0.48	2 ± 0.42	2 ± 0.37	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.01	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.001	1 ± 0.39	1 ± 0.28	1 ± 0.23	1 ± 0.20	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

Symbiotic bacteria	isolate	Extract concen-tration (%)	Mortality rate (%) ± S.D.							
			Aedes aegypti				***Aedes albopictus***			
			24 h	**48 h**	**72 h**	**96 h**	**24 h**	**48 h**	**72 h**	**96 h**
Xenorhabdus indica	bSNK8.5_TH	1	26 ± 4.32	42 ± 4.01	47 ± 3.85	50 ± 3.66	10 ± 3.83	22 ± 4.43	30 ± 3.75	35 ± 3.37
		0.1	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.01	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
		0.001	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
2% DMSO: negative control			0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Dechlorinated water: negative control			0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

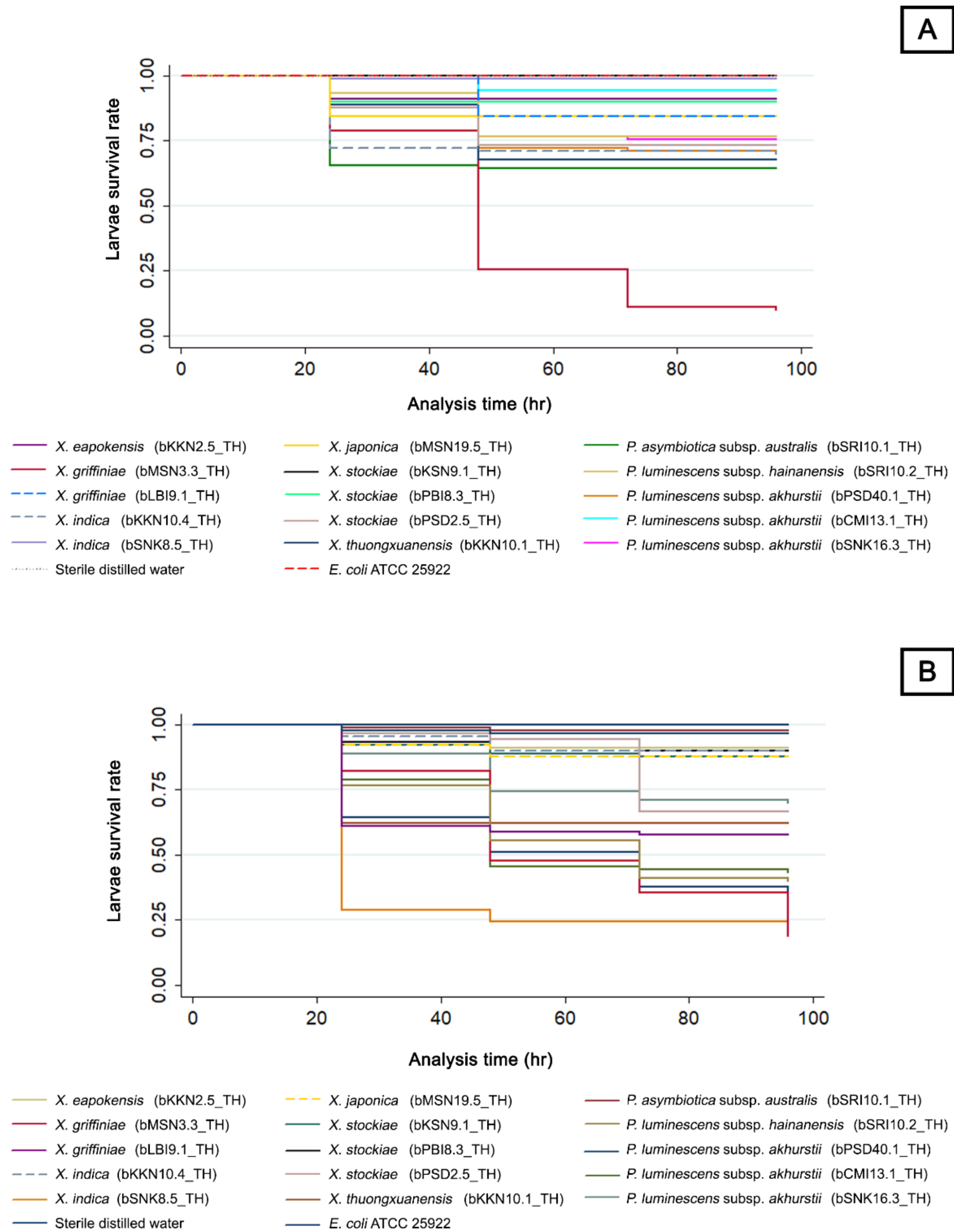


Figure 27 Kaplan–Meier overall survival curve comparing the mortality rates of *Aedes aegypti* (A) and *Aedes albopictus* (B) larvae after exposure to whole-cell suspensions of *Photorhabdus* and *Xenorhabdus* isolated from EPNs in 12 provinces of Thailand

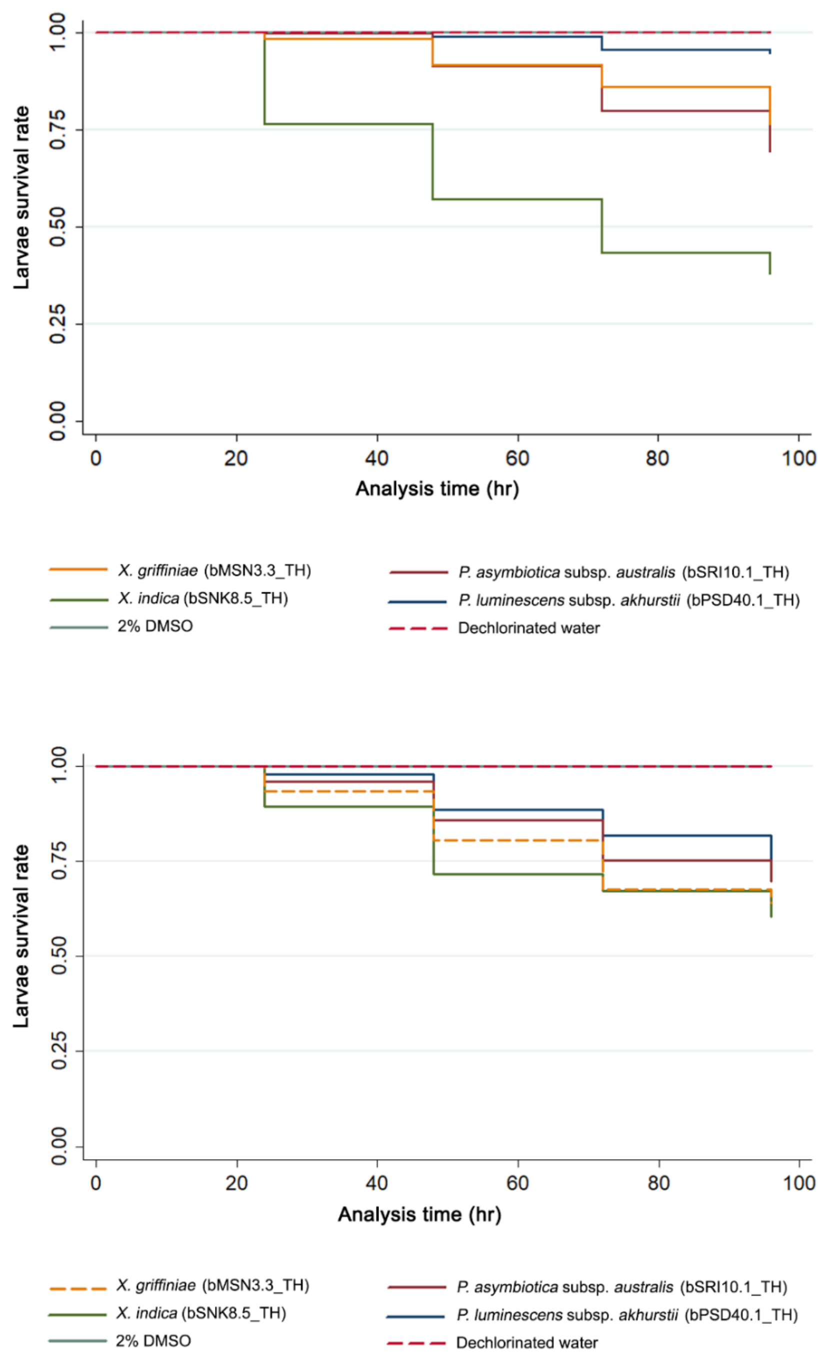


Figure 28 Kaplan–Meier overall survival curve comparing the mortality rates of *Aedes aegypti* (A) and *Aedes albopictus* (B) larvae after exposure to 1 % crude extract solution of *Photorhabdus* and *Xenorhabdus* isolated from EPNs in 12 provinces of Thailand

CHAPTER V

DISCUSSION

In this study, 118 of 1100 soil samples were found for EPNs (10.73% prevalence) in genus *Heterorhabditis* (6.27%) and *Steinernema* (4.46%). *Heterorhabditis indica*, *H. baujardi*, *Heterorhabditis* sp. SGmg3, *S. surkhetense*, *S. kushidai*, *S. guangdongense*, *S. siamkayai*, *S. huense*, *Steinernema* sp. YNd80, and *Steinernema* sp. YNc215 were molecularly identified based on the sequencing of ITS or 28S rDNA. Our findings agree with previous studies on the survey of EPNs in Thailand, which reported the prevalence of EPNs as 2.87–9.10% from different ecologies of Thailand (Ardpairin et al., 2020; Muangpat et al., 2017; Thanwisai et al., 2021; Vitta et al., 2017; Yooyangket et al., 2018). However, this study found higher EPN prevalence compared to the previous surveys. This may be more soil samples were collected from various ecologies such as roadsides, hot springs, and agricultural areas. Most of the EPNs were isolated from loam soil, which was compatible with the previous studies (Ardpairin et al., 2020; Muangpat et al., 2017; Suwannaroj et al., 2020; Vitta et al., 2017; Yooyangket et al., 2018). The appearance of loam soil has more gaps between soil pellets and more oxygen which leading to EPNs movement and survival (Kung et al., 1991). However, a few samples from clay and clay loam soils were found EPN, suggesting that EPNs can survive in various soil textures. The number of *Heterorhabditis* isolates discovered were more than the number of *Steinernema* isolates, which is consistent with the findings of Muangpat et al. (2017) and Suwannaroj et al. (2020).

Heterorhabditis indica had the most EPN isolates (50 isolates) in our study. This nematode was first discovered from sugarcane top borer (*Scirpophaga excerptalis*) in India (Poinar et al., 1992). Subsequently, they are globally distributed, as it is found in Algeria, China, Egypt, Japan, Mexico, Nepal, Pakistan, Saudi Arabia and Vietnam (Bhat, Chaubey, & Askary, 2020). *Heterorhabditis indica* is a common EPN species in Thailand and is usually found in various provinces such as Khon Kaen, Phetchabun, Chiang Mai, Chiang Rai, Nan, Phayao, Phrae, Lampang,

Lamphun, and Mae Hong Son. Therefore, this founding confirms that *H. indica* is a common EPN species in Thailand. *Heterorhabditis indica* has been successfully controlloed various insects. Several strains of this EPN showed highly virulent against the larvae of land and water insects such as the Fall armyworm (*Spodoptera frugiperda*), sweet potato weevil (*Cylas Formicarius elegantulus*) and *Anopheles stephensi* (Acharya, Hwang, Mostafiz, Yu, & Lee, 2020; Dilipkumar et al., 2019; Myers, Sylva, Mello, & Snook, 2020).

Besides, *Heterorhabditis baujardi* and *Heterorhabditis* sp. SGmg3 were also found in this study, which was similar to the finding of Thanwisai et al. (2012) and Yimtin et al. (2021). *Heterorhabditis baujardi* was discovered in Vietnam, Ethiopia, Egypt, and China (Bhat et al., 2020). Their morphology is similar to *H. indica*, except for the shape of the gubernaculum and the number of genital papillae (Phan et al., 2003). *Heterorhabditis baujardi* isolate LPP7 have potential to control the cattle tick (*Rhipicephalus microplus*) (de Mendonça et al., 2019), *Stomoxys calcitrans* (Leal et al., 2017) (Leal, Monteiro, Mendonça, Bittencourt, & Bittencourt, 2017b), and the aquatic snail (*Lymnaea columella*) (Tunholi et al., 2017). For the *Heterorhabditis* sp. SGmg3 isolate found in this study, this nematode isolate was first reported from Meghalaya, India. Based on only the nucleotide sequence of this EPN strain at the National Center for Biotechnology Information (NCBI) (accession no. FJ751864), the taxonomic status of Heterorhabditis sp. SGmg3 is unclear. Nevertheless, this EPN strain was continuously reported from Thailand (Suwannaroj et al., 2020; Thanwisai et al., 2012; Vitta et al., 2017; Yimthin et al., 2021)

Therefore, studying the taxonomy of this nematode is suggested. For genus *Steinernema*, the most identified EPN was *S. surkhetense*, a finding that is in line with Suwannaroj et al. (2020), which recovered *S. surkhetense* from the Phitsanulok province of Thailand. In this study, this nematode was found in loam from various provinces such as Lopburi, Saraburi, Phayao, Uttaradit, Kalasin, Khon Kaen, Sakon Nakhon and Phetchaburi. *Steinernema surkhetense* was first discovered from Nepal (Khatri-Chhetri et al., 2011c). Subsequently, this nematode, reported in the agricultural area of India, was experimentally tested for its virulence on tobacco caterpillar (*Spodoptera litura*) (Bhat, Stkhar, Chaubey, Puza, & San-Blas, 2017). In addution, only one isolate of *S. kushidai* was discovered from the soil sample

collected around Pong Dueat hot spring in the Chiang Mai province, northern Thailand. This nematode was isolated from loam soil with pH 6.6, temperature 26°C, and moisture 1.0. This is related with Muangpat et al. (2017), which found only one isolate of *S. kushidai* from the loam soil in Mae Wong National Park in Kamphaeng Phet province, central Thailand, with pH 7.0, temperature 21°C, and moisture 1.0. This supported that *S. kushidai* has low prevalence in Thailand and is usually found in low humidity soil areas with soil pH ranging from 6.6 to 7.0. *Steinernema kushidai* was first recovered from the cadavers of scarabaeid beetle larva (*Anomala cuprea*) in Japan (Mamiya, 1988). This EPN was reported from China in a similar soil texture with Thailand and also found in sandy loam of vegetable crops (Wang, Luan, Dong, Qian, & Cong, 2014). *Steinernema kushidai* was also reported from Russia and Egypt (Bhat et al., 2020). Thus, this reveals that *S. kushidai* survives in a variety of habitats. Herein, an isolate of *S. siamkayai* was identified, which was recovered from loam soil with pH 7.0, temperature 30°C, and moisture 1.0 in the area of eucalyptus forest in the Khon Kaen province, northeast Thailand. This founding agrees with Stock et al. (1998), Ardpairin et al. (2020) and Khatri-Chhetri et al. (2010), which obtained this nematode species in Phetchabun and Phitsanulok provinces of Thailand and Nepal, respectively. *Steinernema siamkayai* is often found in agricultural areas in loam and sandy loam. It has been reported to be pathogenicity to cotton bollworm (*Helicoverpa armigera*) and tobacco cutworm (*S. litura*) (Bhat, Chaubey, Hartmann, Nermut', & Půža, 2021). Furthermore, this is the first recovered of *S. guangdongense*, *S. huense*, *Steinernema* sp. YNd80, and *Steinernema* sp. YNc215 in Thailand. *Steinernema guangdongense* was first isolated from the sandy loam soil of an artificial eucalypt forest in Guangdong province, China (Qiu et al., 2004). This is relatively in line with this study, which also found this EPN species in the loam soil of the eucalyptus forest. Contrarily, *S. huense* from this finding was observed in the loam soil of the roadside forests of Kalasin province, Thailand, which was different from the previous research that found this species in clay soil from Bach Ma National Park, Vietnam (Phan et al., 2014). Moreover, since *Steinernema* sp. YNd80 and *Steinernema* sp. YNc215 are unclassified species and their biological aspects are unclear, their taxonomic stations or potential to control insect pests should be promptly clarified. There have been no reports about the insect control of all four species.

Entomopathogenic nematode in genus *Heterorhabditis* and *Steinernema* are symbiotically associated with gram negative bacteria in genus *Photorhabdus* and *Xenorhabdus*, respectively. Herein, several species of these bacteria were isolated and identified by partial DNA sequencing of the recombinase A (*recA*) gene which is highly conserved housekeeping gene in bacteria (Bethany, Vincent, & Susan, 2007; Takle, Toth, & Brurberg, 2007) The nucleotide sequences of the *recA* gene contain a high variation and provide correct phylogenetic cluster. Therefore, this gene has sufficient discrimination between *Photorhabdus* and *Xenorhabdus* species (Tailliez et al., 2010).

In the current study, *P. luminescens* subsp. *akhurstii* (n = 41) was the most prevalent *Photorhabdus* isolate, and *X. stockiae* (n = 22) was the most common *Xenorhabdus* isolate. Several *P. luminescens* subsp. *akhurstii* isolates have previously been identified, with 10 to 45 isolates reported in Thailand, while the number of *X. stockiae* isolates reported ranges from 27 to 66 (Fukruksa et al., 2017; Muangpat et al., 2017; Suwannaroj et al., 2020; Thanwisai et al., 2021; Thanwisai et al., 2012; Yimthin et al., 2021; Yooyangket et al., 2018) The high prevalence of *P. luminescens* subsp. *akhurstii* are related with the prevalence of their host, *H. indica*, which is a common EPN species in Thailand (Abd-Elgawad, 2021; Yimthin et al., 2021). The minor *Photorhabdus* and *Xenorhabdus* bacterial isolates obtained in this study, *P. asymbiotica* subsp. *australis*, *X. griffiniae*, *X. japonica*, *X. eapokensis*, and *X. miraniensis*, are agree with the results of previous studies conducted in Thailand. The low prevalence of these bacteria could result from the survival and distribution of EPN hosts in this area. Several abiotic factors, such as soil temperature, soil pH, and moisture affect the survival of these soil-dwelling nematodes (Fukruksa et al., 2017; Muangpat et al., 2017; Suwannaroj et al., 2020; Thanwisai et al., 2021; Thanwisai et al., 2012; Yimthin et al., 2021; Yooyangket et al., 2018).

In this study, three *Photorhabdus* species including *P. luminescens* subsp. *akhurstii*, *P. hainanensis*, and *P. asymbiotica* subsp. *australis* were isolated from *Heterorhabditis* nematodes discovered in soil samples. The symbiotic relationship between *P. luminescens* subsp. *akhurstii* and *H. indica* has been reported in Australia, Cuba, the Dominican Republic, Israel, Jamaica, and Puerto Rico (Abd-Elgawad, 2021; Fischer-Le Saux et al., 1999). *Photorhabdus luminescens* subsp. *akhurstii* was

previously reported relationship with *H. baujardi* and *Heterorhabditis* SGmg3 in Thailand (Fukruksa et al., 2017; Muangpat et al., 2017; Thanwisai et al., 2012; Yimthin et al., 2021; Yooyangket et al., 2018). *Photorhabdus luminescens* subsp. *hainanensis* was first reported from an unidentified *Heterorhabditis* nematode on Hainan Island, China (Tailliez et al., 2010). Our finding showed evidence that *Heterorhabditis indica* is a host for *P. luminescens* subsp. *hainanensis*. Our study found evidence for *Heterorhabditis indica* are host of *P. hainanensis* which in line with previous study of Yimthin et al. (2021) that reported *H. indica* and *Heterorhabditis* sp. SGmg3 are host for *P. luminescens* subsp. *hainanensis*. *Photorhabdus asymbiotica* subsp. *australis* not only infects in insects but also is an opportunistic human pathogen (Hapeshi, & Waterfield, 2017). This bacteria was first isolated from human clinical specimens from four Australian patients (Peel et al., 1999). Subsequently, a bacterium was isolated from *H. indica* and *Heterorhabditis* sp. SGmg3, which were discovered in soil samples of Thailand (Suwannaroj et al., 2020; Thanwisai et al., 2012; Yimthin et al., 2021). *Heterorhabditis indica* and *Heterorhabditis* sp. SGmg3, which are hosted by *P. asymbiotica* subsp. *australis* was also observed in the present study.

Six *Xenorhabdus* species were identified in this study; *X. stockiae*, *X. indica*, *X. griffiniae*, *X. japonica*, *X. thuongxuanensis*, and *X. eapokensis*. In 2006, Tailliez et al. reported the first finding of *X. stockiae* isolated from *S. siamkayai,* a Thai entomopathogenic nematode. *Xenorhabdus stockiae* is also symbiotically associated with *S. surkhetense* from India and Thailand, *S. huense* from Vietnam, and *S. websteri* and *S. siamkayai* from Thailand (Bhat et al., 2017; Fukruksa et al., 2017; Muangpat et al., 2017; Phan et al., 2014; Suwannaroj et al., 2020; Thanwisai et al., 2012; Yimthin et al., 2021; Yooyangket et al., 2018) We have also reported a symbiotic relationship between *X. stockiae* and *S. huense* or *S. surkhetense*. According to phylogenetic relationships, *S. huense* and *S. surkhetens*e belong to the carpocapsae group (Khatri-Chhetri et al., 2011c; Phan et al., 2014). *Xenorhabdus stockiae* has the ability to alternate within nematodes clade. Previous study revealed at least 17 host switches of *Xenorhabdus* spp. strains within and between clades (Lee, & Stock, 2010). *Xenorhabdus indica* is normally found in agricultural fields and has been associated with *S. abbasi* (90 % similarity), *S. thermophilum*, *S. yirgalemense*, and

S. pakistanense. On the other hand, *in this study*, an interdependent partnership was noted between *X. indica* and *S. surkhetense*. The initial identification of *Xenorhabdus griffiniae* occurred within *S. hermaphroditum* in Indonesia. (Tailliez et al., 2006). Moreover, a mutually beneficial association has been documented between *X. griffiniae* and *S. khoisanae*, alongside an unidentified *Steinernema* species originating from South Africa. (Dreyer, Malan, & Dicks, 2018; Mothupi, Featherston, & Gray, 2015). *Xenorhabdus japonica* has been isolated from *S. kushidai* nematodes in both Japan (Nishimura et al., 1994) and Thailand (Muangpat et al., 2017; Yooyangket et al., 2018) The symbiotically relationship between *X. japonica* and *S. kushidai* were also discovered in the present study. The remaining *Xenorhabdus* species found in this study, *X. thuongxuanensis* and *X. eapokensis*, were first reported in association with nematodes from Vietnam, which *X. thuongxuanensis* was associated with *S. sangi* and *X. eapokensis* with *S. eapokense* (Kämpfer et al., 2017). Markedly, *X. eapokensis* has also been recovered from *S. sangi* in northeastern Thailand (Yimthin et al., 2021) and *X. thuongxuanensis* was first isolated from EPNs sourced in China (Kämpfer et al., 2017). In the present study, this bacterium was found to be associated with *S. guangdongense*. The association between *X. thuongxuanensis* and *S. guangdongense* is a new finding in terms of symbiotic relationships.

Steinernema surkhetense isolate ePYO8.5_TH showed high virulent to *Ae. aegypti* larvae in this study. The EPN infection in mosquito larvae was first examined by Welch and Bronskill (1962). They reported 82% mortality of *Ae. aegypti* larvae after exposure to *S. carpocapsae*. In the recent decade, Chaudhary et al. (2017) reported that 100 IJs of *S. kraussei* have ability to kill 100% of *Ae. aegypti* bred in canal, tap, and sewage water at 20°C after 48 and 96 h of exposure. Subsequently, Dilipkumar et al. (2019) reported that *Ae. aegypti* larvae were susceptible to *S. abba*si with a 97% mortality after a 48 h exposure. Recently, Edmunds, Wilding, and Rae (2021) experimentally evaluated the mortality of *Ae. aegypti* and *Ochlerotatus detritus* larvae after exposure to commercial-strain EPNs; *S. feltiae, S. carpocapsae, S. kraussei,* and *H. bacteriophora* and wild-isolated EPNs; *S. affine* and *S. glaseri*. The result showed that the commercial EPN strains had high virulence to *Ae. aegypti* and *O. detritus*, while the wild strains were effective in killing only *O. detritus*. In this

study, *S. surkhetense* at all concentrations (800, 1600, 3200, and 6400 IJs) showed high virulence *to Ae. aegypti* larvae. Furthermore, EPN in genus *Steinernema* ascertained that it is effective against other species in the Culicinae subfamily. Moreover, *Steinernema abbasi* was reported the pathogenic to the third and fourth instar larvae of *Ae. albopictus* treated with 1×10^3 IJs/ml and 1×10^4 IJs/ml (Liu, Chen, Hou, Chen, & Tu, 2020). In addition, *Steinernema siamkayai* was shown to be effective against *Cx. quinquefasciatus* (98.67%) at a 100 IJs/larva concentration at 24 and 48 h (Dilipkumar et al., 2019). Recently, *S. carpocapsae*, the native strain of Mexico, showed high virulence to *Ae. aegypti larvae* (Treviño-Cueto, Subbotin, & Sanchez-Peña, 2021). Thus, EPN in genus *Steinernema* has been significantly virulent to the larvae of the culicine mosquito. Although the highest prevalence of *H. indica* was found here but the virulence to *Ae. aegypti* larvae was low. Dilipkumar et al. (2019) revealed *H. indica* had high potential against *Cx. quinquefasciatus* and *An. stephensi* but low potential against *Ae. aegypti* when treated with 100 IJs/larva at 24 h. However, *H. indica* has been reported successfully to control various insects such as the fall armyworm (*Sp. frugiperda*) (Acharya et al., 2020) and sweet potato weevil (*Cy. formicarius elegantulus*) (Myers et al., 2020).

Heterorhabditis and *Steinernema* nematodes usually penetrate an insect larva through its natural openings for example anus, mouth, and spiracle. Nonetheless, only *Heterorhabditis* have ability to invade via the cuticle cause its terminal tooth (Bedding , & Molyneux, 1982; Burnell , & Stock, 2000). At this time, through the experiments conducted in water, *H. indica* was observed all over inside the body of the *Ae. aegypti* larvae, whereas almost all of *S. surkhetense* was observed in the hemocoel. *Steinernema surkhetense* may first enter through the natural opening and then move to the gastric caecum and pierce the hemocoel, as demonstrated in Liu et al. (2020), which tested on *Ae. albopictus* larvae and *S. abbasi*. Besides, dead EPNs were found in all parts of the *Ae. aegypti* larvae, and alive EPNs were found to be piercing inside the cavity of the mosquito larvae. Therefore, The death of mosquito larvae might due to internal organ damage from EPN penetration. Some black remains of the IJ in the *Ae. aegypti* larvae were also found in this study. Likewise, the black remains of *S. abbasi* inside the body of *Ae. albopictus* was also reported (Liu et al., 2020). This may be the initial cause of the encapsulation. The humoral and

cellular defense of *Ae. aegypti* larvae responds against invading EPN (Chen, & Laurence, 1985). When EPNs invade the mosquito larvae, they are recognized by the immune system of mosquitoes. It influences the synthesis and secretion of antimicrobial peptides (AMPs) from the fat body, the activation of phenoloxidase cascade, the production of melanin, and the encapsulation response by hemocytes around the nematode associated with blackening the capsule (Castillo, Reynolds, & Eleftherianos, 2011). The IJ stages of EPNs are free-living stages that survive outside the host. This stage is adapted to have energy sources such as lipid, fatty acids, and glycogen for living in an environment without feeding while searching for a new insect host as a food source (Glazer, 2001). Therefore, the entomopathogenic nematodes have primarily faced soil-dwelling insects (Arthurs, Heinz, & Prasifka, 2004; Belien, 2018; Lacey, & Georgis, 2012). In dry soil, the IJ stage can persist for 2 to 3 weeks (Kaya, 1990; Kung, Gaugler, & Kaya, 1990). Nevertheless, Edmunds, Wilding, & Rae (2017) suggested that *S. feltiae*, *S. carpocapsae, S. kraussei*, and *H. bacteriophora* were able to kill an aquatic larval stage of buzzer midge (*Chironomus plumosus*) with less than 20% survival after four days. More than 96% of EPNs can endure in water (a depth of 30 cm) up to 96 h. Moreover, several studies explained that EPNs have longevity in water and still retain the ability to infect. For example, the infective juvenile of *S. abbasi* and *H. indica* had 85.76% and 88.09% survival rate at 30°C after 15 days of exposure, respectively (Sunanda, Siddiqui, & Sharma, 2012). *Heterorhabditis indica* can be kept in distilled water at 8°C and 30°C for 42 days, while *S. bicornutum* can be stored only at 8°C, and the storage in low temperature is not affect to their virulence against *G. mellonella* (Hussaini, Singh, Parthasarathy, & Shakeela, 2000). After 90 days of incubation under controlled laboratory conditions, *H. indica* showed a survival rate of 77.29%, 61.30%, and 54.60% at 20°C, room temperature (25–28°C), and 30°C, respectively (Amit, Vijaya, Sunanda, & Vinod, 2017). Naturally, the *Aedes* larvae feed by swimming or diving to the target food sunk at the bottom and catch tiny organisms with their lateral palatal brushes or mouth-brush (Merritt, Dadd, & Walker, 1992). Therefore, although EPNs sink to the bottom of the container, *Aedes* larvae attempt to eat them, which leads to invade through the mouth. Treviño-Cueto et al. (2021) emphasized this strategy that observed numerous IJs in the head capsule of infected *Aedes* larvae. Additionally,

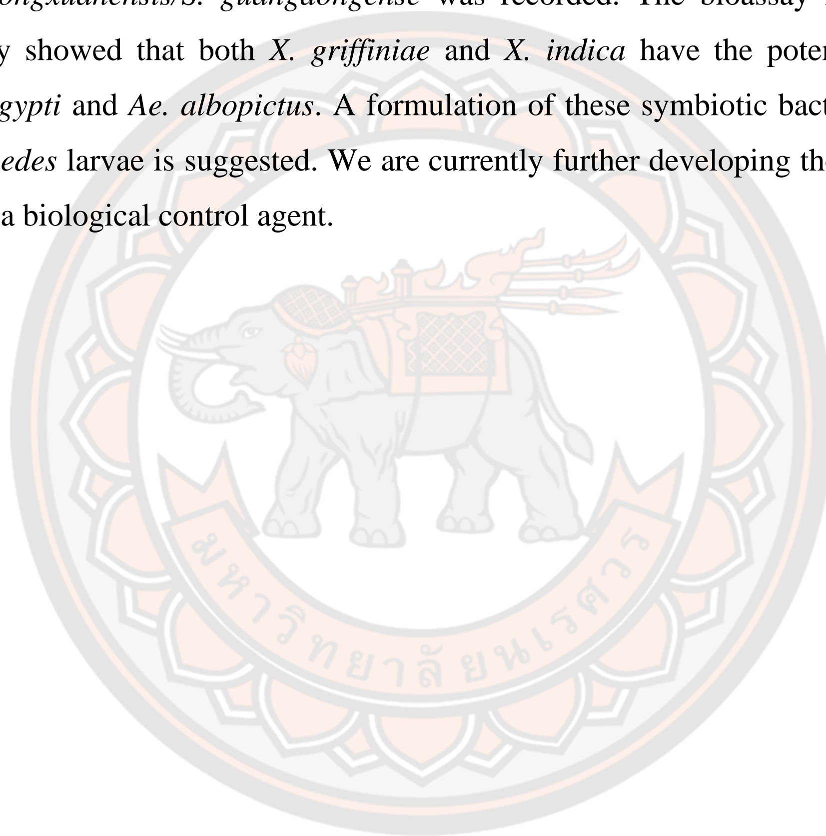
Aedes has various breeding sites such as jars, tanks, and abandoned objects, and the impact of water volume and water depth on the efficacy of EPNs against *Ae. aegypti* was studied by Shan et al. (2021). However, no significant differences in the mortality of *H. bacteriophora* were revealed after four days. From previous studies, using EPNs to get rid of aquatic organisms such as mosquito larvae is expected to be possible, and the results of this study refer to 88% mortality of *Ae. aegypti* larvae within four days. Hence, an EPN could potentially be an alternative bio-control agent for *Ae. aegypti* larvae.

In this study found that *X. griffiniae* bMSN3.3_TH showed potential in eradicating both *Ae. aegypti* and *Ae. albopictus* larvae, with 80–90% mortality after 96 h exposure. This finding is in agreement with that of a previous study by Thanwisai et al. (2021), who reported high mortality (91%) in *Ae. aegypti* larvae after exposure to *X. griffiniae* (bKCK26.3_TH). The high mortality of *Aedes* spp. may be due to the bioactive compounds that are produced by this bacterium. Kim et al. (2017) reported that *X. innexi* releases the *Xenorhabdus* lipoprotein toxin (Xlt), which has the ability to create pores on cells in the midgut of mosquitoes, leading to cell death. Several previous studies have provided evidence for metabolites that are potent in controlling culicid mosquitoes, such as benzylideneacetone, iodine, phenethylamides, indol derivatives, xenorhabdins, xenooxides, and xenocoumacins (da Silva, Pilz-Júnior, Heermann, & da Silva, 2020). Some *Xenorhabdus* strains produce toxin complexes (Tcs) that inhibit eicosanoid synthesis, leading to immunosuppression in insects (da Silva et al., 2020). Eom, Park, and Kim (2014) revealed that *X. nematophila* releases various suppressor metabolites that affect insect immunity. *Photorhabdus luminescens* subsp. *hainanensis* bSRI10.2_TH, *P. luminescens* subsp. *akhurstii* bPSD40.1_TH, and bCMI13.1_TH also showed moderate potential to eliminate *Ae. albopictus* larvae with 57–66% mortality. Rodou, Ankrah, & Stathopoulos (2010) reported that *P. luminescens* establishes Tcs that destroy epithelial cells in the middle intestines of insects. The “make caterpillars floppy” (Mcf) toxin, which is produced by *P. temperata* subsp. *temperate* strain K122 activates hemocyte apoptosis in the hemocoel of insects. *Photorhabdus* virulence cassettes (Pvc) and insect-related protein (Pir), which are released by some strains of *Photorhabdus* bacteria, are also toxic to insects (Rodou et al., 2010). The low

pathogenicity observed against *Aedes* spp. for some strains of symbiotic bacteria in the present study may be a result of low production/efficacy of secondary metabolites. In addition, *Photorhabdus* and *Xenorhabdus* bacteria have “phase variation” features, with bacteria shifting from Phase I, which is associated with the production of a wide range of effective secondary metabolites, to Phase II, with no or weak virulence. This phenomenon can occur during prolonged periods of bacterial culture, extended liquid subculture, or in bacteria living outside the nematode (Forst, & Clarke, 2002; Pinyon, Hew, & Thomas, 2000). In terms of mortality, the whole cell suspension was more effective than bacterial extracts against *Aedes* larvae. This might be due to the fact that whole cell suspensions contain living bacterial cells, which might be able to multiply within the gut of mosquito larvae and continue to produce bioactive compounds that are active against mosquito larvae. In terms of the ethyl acetate extract, the game peptide derivatives, isopropyl stilbene, xenoamicin derivatives, xenocoumacin derivatives, mevalagmapeptide, and phurealipid derivatives dissolved in the extracts of *Xenorhabdus/Photorhabdus* (Muangpat et al., 2017) may have weak insecticidal activity against *Aedes* spp. Although the pathogenicity of *Xenorhabdus* and *Photorhabdus* bacteria in humans has not been reported, except for *Photorhabdus asymbiotica*, the adding of whole cell bacteria directly into the water seems inappropriate in daily life. Because these bacteria are still alive and may cause unexpected effects on aquatic organisms. To apply *Xenorhabdus/Photorhabdus* bacteria for the control of *Aedes* mosquitoes, additional processes such as the purification and identification of effective metabolites and toxicity testing in the environment and for other organisms are necessary.

In conclusion, the following EPN species were molecularly identified from the 12 provinces across Thailand: *H. indica, S. surkhetense, S. kushidai, S. siamkayai, H. baujardi,* and *Heterorhabditis* sp. SGmg3. According to our survey, *Steinernema* sp. YNd80, *Steinernema* sp. YNc215, *S. guangdongense*, and *S. huense* have been identified for the first time, and *H. indica* is a common species in Thailand. In addition, the *Steinernema surkhetense* ePYO8.5_TH isolate showed the potential to kill *Aedes aegypti* larvae. Therefore, it is possible to apply the entomopathogenic nematode, *Steinernema surkhetense* ePYO8.5_TH, as a biological control agent to control *Ae. aegypti* larvae. This EPN isolate offers high mortality of the mosquito

larvae and is also environmentally friendly, thus reducing the risk of mosquito repellent resistance. we report the identification of *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*, *P. asymbiotica* subsp. *australis*, *X. stockiae*, *X. indica, X. griffiniae, X. japonica, X. thuongxuanensis*, and *X. eapokensis* in isolates of EPNs from Thailand. *Xenorhabdus thuongxuanensis*, isolated from *S. siamkayai* and *S. guangdongense*, is the first record of symbiotic bacteria in Thailand. The first instance of a symbiotic relationship between *X. indica/S. surkhetense* and *X. thuongxuanensis/S. guangdongense* was recorded. The bioassay for insecticidal activity showed that both *X. griffiniae* and *X. indica* have the potential to control *Ae. aegypti* and *Ae. albopictus*. A formulation of these symbiotic bacteria for testing with *Aedes* larvae is suggested. We are currently further developing these bacteria for use as a biological control agent.



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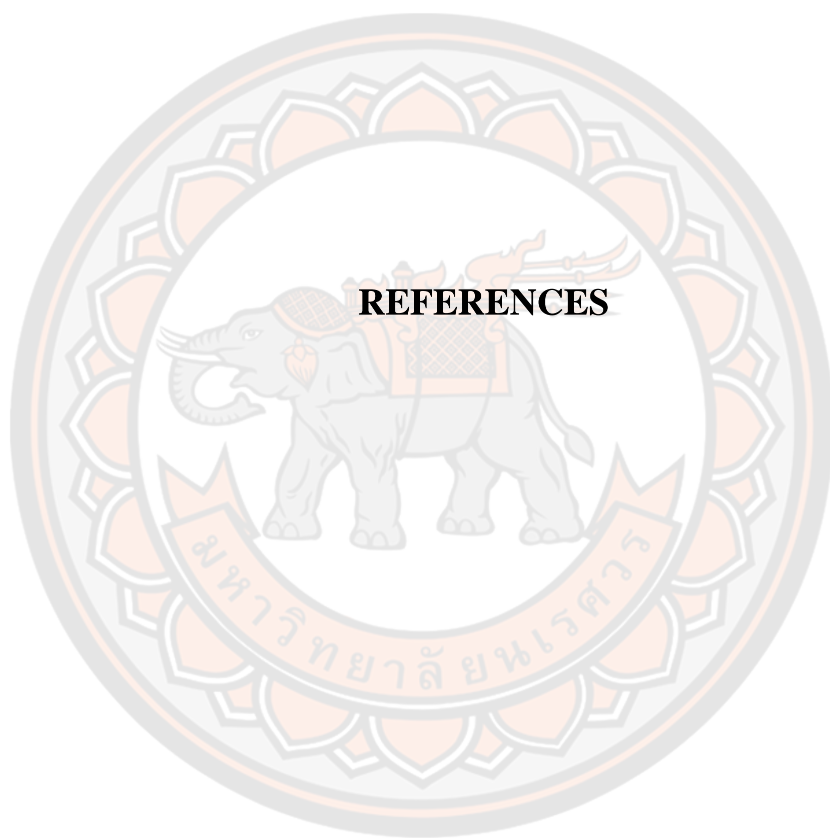

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APPENDIX

APPENDIX A CULTURE MEDIUM AND CHEMICALS PREPARATION

Nutrient- bromothymol blue- triphenyl tetazolium chioride agar (NBTA)

Components for 1 L

Nutrient agar (Oxoid, Ltd, England) 28 g

Bromothymol blue 0.025 g

Filtered 0.004% tetrazolium chloride 500 µL (dissolved with 90% alcohol, 0.22 µm filter)

Distilled water 1 L

Preparation

Dissolve all component with distilled water in flask, then sterilize by adding the flask into autoclave at 121 °C for 15 min. After that, let the medium in flask cool down until around 50°C, follow by adding tetrazolium chloride. Lastly, pouring the medium into sterilized petri dish. Let the medium cool down until become to an agar. The NBTA is ready to use or keep in 4°C fridge.

Luria-Bertani broth (LB)

Components for 1 L

Luria-Bertani broth (LB) powder (Caisson LABS, USA) 25 g

Distilled water 1 L

Preparation

Dissolve LB powder with distilled water in flask, then sterilize by adding the flask into autoclave at 121 °C for 15 min. After that, let the medium in flask cool down. The LB is ready to use or keep in 4°C fridge.

Tryptone soy agar (TSA)

Components for 1 L

Tryptone soya agar (TSA) (Oxoid, Ltd, England) 40 g

Distilled water 1 L

Preparation

Dissolve TSA with distilled water in flask, then sterilize by adding the flask

into autoclave at 121 °C for 15 min. After that, let the medium in flask cool down. The LB is ready to use or keep in 4°C fridge.

5YS broth

Components for 1 L

Yeast extract	50	g
NaCl	5	g
K_2HPO_4	0.5	g
$NH_4H_2PO_4$	0.5	g
$MgSO_4 \cdot 7H_2O$	0.2	g
Distilled water	1	L

Preparation

Dissolve all components with distilled water in flask, then sterilize by adding the flask into autoclave at 121 °C for 15 min. After that, let the broth in flask cool down. The 5YS brith is ready to use or keep in 4°C fridge.

10X TBE buffer

Components for 1 L

Tris base (Oxoid, Ltd, England)	108	g
Boric acid	55	g
Ethylenediaminetetraacetic acid (EDTA)	7.5	g
Distilled water	1	L

Preparation

Dissolve all component with 800 mL distilled water in flask, then adjust the volume until 1 L. The 10X TBE is ready to use or keep in RT.

1.2% agarose gel

Components for 100 ml

Agarose powder	1.2	g
1X TBE buffer	100	ml

Preparation

Dissolve agarose powder with 1X TBE buffer in flask, then heat by adding the flask in to microwave for 1 – 1.30 min. Let the dissolved gel cool down a little bit in RT and pour to gel tray.

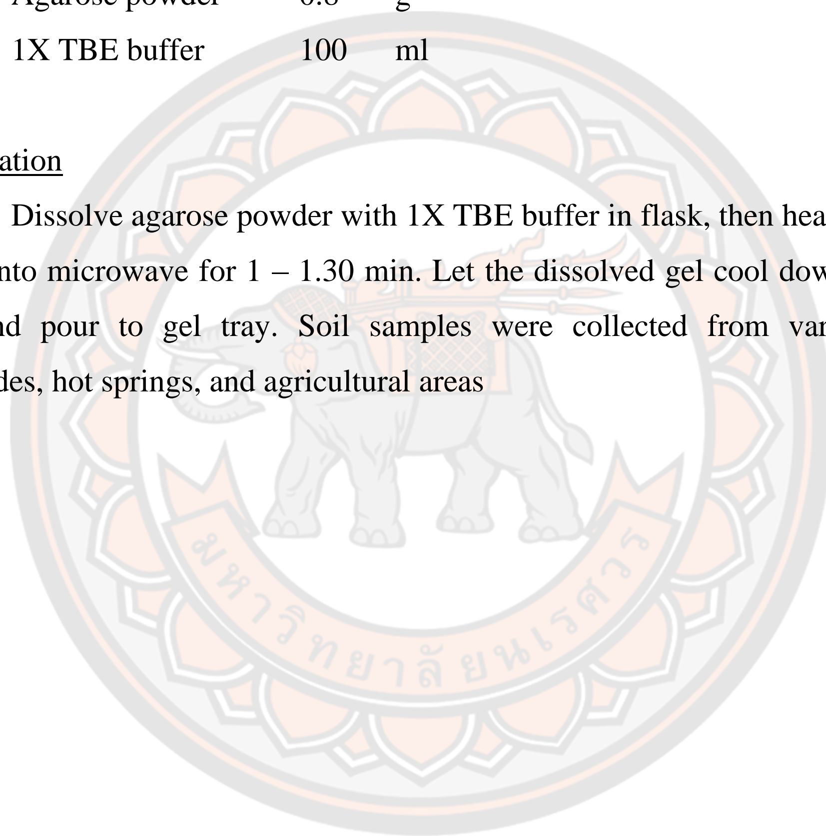
0.8% agarose gel

Components for 100 ml

Agarose powder	0.8	g
1X TBE buffer	100	ml

Preparation

Dissolve agarose powder with 1X TBE buffer in flask, then heat by putting the flask into microwave for 1 – 1.30 min. Let the dissolved gel cool down a little bit in RT and pour to gel tray. Soil samples were collected from various ecologies roadsides, hot springs, and agricultural areas



APPENDIX B SOIL SAMPLE COLLECTION IN THAILAND

Figure 29 Soil sample collection nearby National Park areas

Figure 30 Soil sample collection at road side areas

Figure 31 Soil sample collection at agricultural areas

Figure 32 Extrinsic factors and geographic coordinates measurement

APPENDIX C SOIL SAMPLE INFORMATION

Table 23 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Chiang Mai province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	9.25 a.m.	1	1	N 19° 15’ 04.3”	6.8	24	1	Loam	Not found
			2	E 098° 38’ 16.7”	7	24	1	Loam	Not found
			3		6.8	24	1	Loam	Not found
			4	Elev = 1189 m	7	23	1	Loam	Not found
			5		6.8	23	1	Loam	Not found
17/6/2018	9.30 a.m.	2	1	N 19° 15’ 03.5”	6.6	24	1	Loam	Not found
			2	E 098° 38’ 168.6"	6.8	24	1	Loam	Not found
			3		7	24	1	Loam	Not found
			4	Elev = 1181 m	7	24	1	Loam	Not found
			5		7	24	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	9.35 a.m.	3	1	N 19° 14’ 35.5”	6.2	27	2	Loam	Not found
			2	E 098° 41’ 02.7”	6.8	26	1	Loam	Not found
			3		6.6	26	1	Loam	Found
			4	Elev = 780 m	6.6	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found
17/6/2018	9.40 a.m.	4	1	N 19° 14’ 34.2”	6.4	26	2	Loam	Not found
			2	E 098° 41’ 04.0”	6.6	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 808 m	6.6	25	1	Loam	Not found
			5		6.8	25	1	Loam	Not found
17/6/2018	9.45 a.m.	5	1	N 19° 14’ 38.2”	6.8	26	1	Loam	Not found
			2	E 098° 41’ 03.3”	6.8	25	1	Loam	Not found
			3		6.8	25	1	Loam	Not found
			4	Elev = 766 m	6.8	25	1	Loam	Not found
			5		6.6	25	2	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	9.50 a.m.	6	1	N 19° 14’ 37.9”	7	25	1	Loam	Not found
			2	E 098° 41’ 03.7”	7	26	1	Loam	Not found
			3		6.8	26	1	Loam	Found
			4	Elev = 770 m	6.8	26	1	Loam	Not found
			5		7	26	1	Loam	Not found
17/6/2018	9.55 a.m.	7	1	N 19° 14’ 38.2”	5	29	6	Loam	Not found
			2	E 098° 41’ 06.1”	6.8	29	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 783 m	6.6	29	2	Loam	Not found
			5		6.4	29	2	Loam	Not found
17/6/2018	10.00 a.m.	8	1	N 19° 14’ 38.2”	6.8	27	1	Loam	Not found
			2	E 098° 41’ 05.4”	6.8	27	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 791 m	6.8	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	10.05 a.m.	9	1	N 19° 14’ 40.5”	6.8	26	1	Loam	Not found
			2	E 098° 41’ 07.4”	6.8	25	2	Loam	Not found
			3		6.8	25	3	Loam	Not found
			4	Elev = 795 m	6.8	25	1	Loam	Not found
			5		6.8	25	1	Loam	Not found
17/6/2018	10.10 a.m.	10	1	N 19° 14’ 39.0”	6.8	27	1	Loam	Not found
			2	E 098° 41’ 06.6”	6.8	26	1	Loam	Found
			3		6.8	26	1	Loam	Not found
			4	Elev = 788 m	6.4	26	2	Loam	Not found
			5		6.8	26	1	Loam	Not found
17/6/2018	10.15 a.m.	11	1	N 19° 14’ 43.0”	6.8	26	1	Loam	Not found
			2	E 098° 41’ 08.5”	6.8	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 786 m	6.6	25	2	Loam	Not found
			5		6.4	25	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	10.20 a.m.	12	1	N 19° 14’ 41.2”	6.6	25	2	Loam	Found
			2	E 098° 41’ 07.6”	6.6	25	2	Loam	Not found
			3		6.6	25	1	Loam	Not found
			4	Elev = 800 m	6.4	25	2	Loam	Not found
			5		6.8	25	1	Loam	Not found
17/6/2018	10.25 a.m.	13	1	N 19° 14’ 43.8”	6.8	27	1	Loam	Found
			2	E 098° 41’ 10.0”	6.8	27	1	Loam	Not found
			3		6.8	27	2	Loam	Not found
			4	Elev = 781 m	6.8	27	1	Loam	Not found
			5		7	26	1	Loam	Not found
17/6/2018	10.30 a.m.	14	1	N 19° 14’ 43.7”	6.8	25	1	Loam	Found
			2	E 098° 41’ 09.5”	6.8	25	1	Loam	Not found
			3		7	25	1	Loam	Not found
			4	Elev = 788 m	6.8	25	1	Loam	Found
			5		6.8	25	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	10.35 a.m.	15	1	N 19° 14’ 43.9”	6.8	26	1	Loam	Not found
			2	E 098° 41’ 10.3”	6.8	26	1	Loam	Not found
			3		7	26	1	Loam	Found
			4	Elev = 783 m	6.8	26	1	Loam	Found
			5		6.8	26	1	Loam	Not found
17/6/2018	10.40 a.m.	16	1	N 19° 14’ 43.7”	7	26	1	Loam	Not found
			2	E 098° 41’ 11.2”	7	26	1	Loam	Not found
			3		7	26	1	Loam	Not found
			4	Elev = 780 m	6.8	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found
17/6/2018	10.45 a.m.	17	1	N 19° 14’ 43.9”	6.8	27	1	Loam	Not found
			2	E 098° 41’ 09.5”	6.8	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 781 m	6.8	26	1	Loam	Not found
			5		7	26	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
17/6/2018	10.50 a.m.	18	1	N 19° 14’ 42.8”	6.8	26	1	Loam	Not found
			2	E 095° 41’ 12.9”	6.6	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 782 m	6.6	26	1	Loam	Not found
			5		6.8	25	1	Loam	Not found
17/6/2018	10.55 a.m.	19	1	N 19° 14’ 41.3”	6.8	26	1	Loam	Not found
			2	E 098° 41’ 15.9”	6.8	26	1	Loam	Not found
			3		6.8	25	1	Loam	Not found
			4	Elev = 786 m	6.6	25	1	Loam	Not found
			5		6.8	25	1	Loam	Not found
17/6/2018	11.00 a.m.	20	1	N 19° 14’ 40.4”	6.8	27	1	Loam	Not found
			2	E 098° 41’ 17.9”	6.6	28	1	Loam	Not found
			3		6.6	28	1	Loam	Not found
			4	Elev = 761 m	6.6	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found

Table 24 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Kalasin province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	11.12 a.m.	1	1	N 16° 48’ 40.7”	6.8	25	1	Loam	Not found
			2	E 103° 53’ 21.0”	6.6	25	1	Loam	Not found
			3		6.8	25	1	Loam	Not found
			4	Elev = 522 m	6.8	26	1	Loam	Not found
			5		7	26	1	Loam	Not found
3/6/2018	11.18 a.m.	2	1	N 16° 48’ 09.1”	6.8	26	1	Loam	Not found
			2	E 103° 52’ 31.0”	6.8	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 469 m	6.6	26	1	Loam	Not found
			5		6.6	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	11.20 a.m.	3	1	N 16° 48’ 09.7”	6.6	27	1	Loam	Found
			2	E 103° 52’ 31.8”	6.8	27	1	Loam	Not found
			3		6.4	26	1	Loam	Not found
			4	Elev = 505 m	6.8	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found
3/6/2018	11.24 a.m.	4	1	N 16° 48’ 10.4”	6.8	26	1	Loam	Not found
			2	E 103° 52’ 29.1”	6.8	27	1	Loam	Not found
			3		6.8	26	2	Loam	Not found
			4	Elev = 462 m	6.6	28	1	Loam	Not found
			5		6.2	28	1	Loam	Not found
3/6/2018	11.30 a.m.	5	1	N 16° 48’ 36.3”	6.6	27	1	Loam	Not found
			2	E 103° 51’ 27.05”	6.8	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 460 m	6.8	26	1	Loam	Not found
			5		6.6	26	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	11.39 a.m.	6	1	N 16° 47’ 36.3”	6.8	28	1	Loam	Not found
			2	E 103° 51’ 27.5”	7	28	1	Loam	Not found
			3		7	27	1	Loam	Not found
			4	Elev = 372 m	6.4	27	1	Loam	Not found
			5		6.8	27	1	Loam	Not found
3/6/2018	11.54 a.m.	7	1	N 16° 46’ 28.8”	6.8	30	1	Loam	Not found
			2	E 103° 49’ 47.3”	6.8	28	1	Loam	Not found
			3		6.6	28	1	Loam	Not found
			4	Elev = 207 m	6.8	30	1	Loam	Not found
			5		7	30	1	Loam	Not found
3/6/2018	12.00 p.m.	8	1	N 16° 46’ 27.9”	6.6	29	1	Loam	Not found
			2	E 103° 49’ 46.1”	6.8	28	1	Loam	Found
			3		6.8	29	1	Loam	Not found
			4	Elev = 202 m	6.8	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	1.57 p.m.	9	1	N 16° 24’ 19.9”	7	29	1	Loam	Found
			2	E 103° 25’ 18.5”	7	29	1	Loam	Found
			3		7	29	1	Loam	Not found
			4	Elev = 167 m	7	29	1	Loam	Found
			5		6.8	29	1	Loam	Not found
3/6/2018	2.00 p.m.	10	1	N 16° 24’ 19.9”	7	29	1	Loam	Not found
			2	E 103° 25’ 17.4”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 173 m	7	29	1	Loam	Not found
			5		7	29	1	Loam	Not found

Table 25 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Khon Kaen province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	3.48 p.m.	1	1	N 16° 16’ 37.3”	7	28	1	Loam	Not found
			2	E 102° 47’ 01.0”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 178 m	6.8	28	1	Loam	Not found
			5		7	29	1	Loam	Found
3/6/2018	3.57 p.m.	2	1	N 16° 16’ 38.1”	7	28	1	Loam	Found
			2	E 102° 47’ 02.0”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 171 m	7	28	1	Loam	Found
			5		7	28	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	4.02 p.m.	3	1	N 16° 15’ 12.9”	6.6	28	1	Sandy loam	Found
			2	E 102° 46’ 29.0”	7	29	1	Loam	Not found
			3		6.2	29	1	Loam	Not found
			4	Elev = 168 m	6.8	28	1	Loam	Not found
			5		6.8	29	1	Loam	Not found
3/6/2018	4.07 p.m.	4	1	N 16° 15’ 13.9”	6.8	29	1	Loam	Found
			2	E 102° 46’ 29.5”	6.8	28	1	Loam	Found
			3		6.7	28	1	Loam	Not found
			4	Elev = 168 m	6.8	28	1	Loam	Not found
			5		6.8	29	1	Loam	Not found
3/6/2018	4.10 p.m.	5	1	N 16° 15’ 42.3”	7	30	1	Loam	Not found
			2	E 102° 46’ 38.4”	7	30	1	Loam	Not found
			3		7	29	1	Loam	Found
			4	Elev = 165 m	7	30	1	Loam	Not found
			5		7	30	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	4.15 p.m.	6	1	N 16° 15’ 41.3”	7	29	1	Loam	Not found
			2	E 102° 46’ 37.7”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 166 m	7	29	1	Loam	Not found
			5		7	28	1	Loam	Not found
3/6/2018	4.20 p.m.	7	1	N 16° 16’ 19.2”	6.8	28	1	Loam	Not found
			2	E 102° 46’ 52.7”	6.8	29	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 166 m	6.8	29	1	Loam	Not found
			5		6.8	29	1	Loam	Not found
3/6/2018	4.25 p.m.	8	1	N 16° 16’ 18.1”	6.8	28	1	Loam	Not found
			2	E 102° 46’ 52.3”	6.8	28	1	Loam	Found
			3		7	28	1	Loam	Found
			4	Elev = 167 m	7	28	1	Loam	Not found
			5		6.8	28	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	4.30 p.m.	9	1	N 16° 16’ 46.2”	6.8	28	1	Loam	Not found
			2	E 102° 47’ 03.4”	6.8	28	1	Loam	Not found
			3		6.8	28	1	Loam	Not found
			4	Elev = 165 m	7	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found
3/6/2018	4.35 p.m.	10	1	N 16° 16’ 47.0”	6.8	28	1	Loam	Found
			2	E 102° 47’ 03.2”	6.8	28	1	Loam	Not found
			3		7	28	1	Loam	Found
			4	Elev = 163 m	7	28	1	Loam	Found
			5		7	28	1	Loam	Found

Table 26 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Lopburi province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	9.57 a.m.	1	1	N 14° 48’ 13”	6	29	3	Loam	Not found
			2	E 100° 45’ 31”	5	28	5	Loam	Not found
			3		5	28	4	Loam	Not found
			4	Elev = 90 m	4.6	28	7	Loam	Not found
			5		4.8	28	6	Loam	Not found
11/6/2018	10.02 a.m.	2	1	N 14° 48’ 10”	6.2	30	2	Loam	Not found
			2	E 100° 45’ 29”	6.4	30	1	Loam	Not found
			3		6.4	30	1.5	Loam	Not found
			4	Elev = 60 m	6.4	30	1	Loam	Not found
			5		6.6	29	2	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	10.07 a.m.	3	1	N 14° 48’ 9”	4.6	28	3	Loam	Not found
			2	E 100° 45’ 28”	4.4	28	5	Loam	Not found
			3		4.8	28	6	Loam	Not found
			4	Elev = 60 m	4.4	28	7	Loam	Found
			5		4.6	28	7	Loam	Not found
11/6/2018	10.12 a.m.	4	1	N 14° 48’ 15”	6.6	29	1	Loam	Not found
			2	E 100° 45’ 35”	6.4	29	1	Loam	Not found
			3		6.4	29	1	Loam	Not found
			4	Elev = 60 m	6.2	29	2	Loam	Not found
			5		6.2	29	1	Loam	Not found
11/6/2018	10.17 a.m.	5	1	N 14° 48’ 13”	6.8	28	1	Loam	Not found
			2	E 100° 46’ 53”	6.8	29	1	Loam	Not found
			3		6.8	29	1	Loam	Not found
			4	Elev = 80 m	6.8	29	1	Loam	Not found
			5		6.8	29	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	10.22 a.m.	6	1	N 14° 48’ 20”	6.2	30	1	Loam	Not found
			2	E 100° 46’ 51”	6.4	29	2	Loam	Found
			3		6.8	29	1	Loam	Not found
			4	Elev = 60 m	6	29	2	Loam	Not found
			5		6.2	29	4	Loam	Not found
11/6/2018	10.27 a.m.	7	1	N 14° 50’ 18”	6.4	29	1	Loam	Not found
			2	E 100° 50’ 24”	6.8	30	1	Loam	Not found
			3		6.8	31	1	Loam	Found
			4	Elev = 80 m	6.4	31	1	Loam	Not found
			5		6.8	31	1	Loam	Not found
11/6/2018	10.32 a.m.	8	1	N 14° 50’ 16”	5	31	1	Loam	Not found
			2	E 100° 50’ 23”	6.6	32	1	Loam	Not found
			3		5.8	32	1	Loam	Not found
			4	Elev = 90 m	6.4	31	1	Loam	Not found
			5		6.4	31	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	10.37 a.m.	9	1	N 14° 57’ 16”	7	29	1	Loam	Found
			2	E 100° 53’ 53”	7	29	1	Loam	Found
			3		7	29	1	Loam	Not found
			4	Elev = 80 m	6.8	28	1	Loam	Found
			5		6.8	28	1	Loam	Found
11/6/2018	10.42 a.m.	10	1	N 14° 57’ 15”	6.8	29	1	Loam	Not found
			2	E 100° 53’ 55”	6.8	30	1	Loam	Not found
			3		6.8	29	1	Loam	Not found
			4	Elev = 120 m	6.8	30	1	Loam	Not found
			5		6.8	30	1	Loam	Not found
11/6/2018	10.47 a.m.	11	1	N 14° 58’ 19”	7	29	1	Loam	Found
			2	E 100° 54’ 5”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 120 m	6.8	29	1	Loam	Not found
			5		6.8	29	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	10.52 a.m.	12	1	N 14° 58’ 18”	7	29	1	Loam	Not found
			2	E 100° 54’ 5”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 150 m	6.8	29	1	Loam	Not found
			5		7	29	1	Loam	Not found
11/6/2018	10.57 a.m.	13	1	N 15° 1’ 30”	6.8	29	1	Loam	Not found
			2	E 100° 56’ 55”	6.8	32	1	Loam	Not found
			3		6.8	29	1	Loam	Not found
			4	Elev = 60 m	6.6	30	1	Loam	Not found
			5		6.6	30	1	Loam	Not found
11/6/2018	11.02 a.m.	14	1	N 15° 1’ 30”	6.8	31	1	Loam	Not found
			2	E 100° 56’ 55”	6.6	31	1	Loam	Not found
			3		6.8	31	1	Loam	Not found
			4	Elev = 60 m	6.4	31	1	Loam	Not found
			5		6.8	31	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	11.07 a.m.	15	1	N 15° 1’ 57”	6.4	30	1	Loam	Not found
			2	E 100° 57’ 0”	6.6	30	1	Loam	Not found
			3		6.4	30	1	Loam	Found
			4	Elev = 70 m	6	30	1	Loam	Not found
			5		6.6	30	1	Loam	Not found
11/6/2018	11.12 a.m.	16	1	N 15° 1’ 58”	6.6	31	3	Loam	Not found
			2	E 100° 57’ 1”	6.8	31	1	Loam	Not found
			3		6.8	31	1	Loam	Not found
			4	Elev = 70 m	6.6	32	1	Loam	Not found
			5		6.8	31	1	Loam	Not found
11/6/2018	11.17 a.m.	17	1	N 15° 1’ 28”	6.6	30	2	Loam	Not found
			2	E 100° 56’ 56”	6.6	31	2	Loam	Not found
			3		6.2	31	1	Loam	Not found
			4	Elev = 60 m	6.4	30	1	Loam	Not found
			5		6.8	30	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
11/6/2018	11.22 a.m.	18	1	N 15° 2’ 17”	6.8	30	1	Loam	Not found
			2	E 100° 26’ 36”	6.8	30	1	Loam	Not found
			3		6.8	30	1	Loam	Not found
			4	Elev = 60 m	6.6	30	1	Loam	Not found
			5		6.6	30	2	Loam	Not found
11/6/2018	11.27 a.m.	19	1	N 15° 3’ 44”	7	32	1	Loam	Not found
			2	E 100° 58’ 33”	7	32	1	Loam	Not found
			3		6.8	32	1	Loam	Not found
			4	Elev = 60 m	6.8	32	1	Loam	Not found
			5		6.8	32	1	Loam	Not found
11/6/2018	11.32 a.m.	20	1	N 15° 4’ 11”	6.8	31	1	Loam	Not found
			2	E 100° 58’ 45”	6.8	31	1	Loam	Not found
			3		6.4	32	1	Loam	Not found
			4	Elev = 60 m	6.8	31	1	Loam	Not found
			5		6.8	31	1	Loam	Not found

Table 27 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Mae Hong Son province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	5.46 p.m.	1	1	N 19° 18’ 31.0”	6.8	25	1	Loam	Not found
			2	E 098° 28’ 24.6”	6.8	25	1	Loam	Found
			3		6.8	25	1	Loam	Not found
			4	Elev = 513 m	6.8	25	1	Loam	Not found
			5		6.8	25	1	Loam	Not found
16/6/2018	5.51 p.m.	2	1	N 19° 18’ 30.9”	6.8	26	1	Loam	Not found
			2	E 98° 28’ 25.8”	6.6	26	1	Loam	Not found
			3		6.6	26	1	Loam	Not found
			4	Elev = 504 m	6.4	26	1	Loam	Not found
			5		5.2	26	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	5.56 p.m.	3	1	N 19° 18’ 30.3”	6.8	26	1	Loam	Not found
			2	E 098° 28’ 26.9”	6.8	26	1	Loam	Not found
			3		5.8	26	1	Loam	Found
			4	Elev = 502 m	6.8	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found
16/6/2018	6.01 p.m.	4	1	N 19° 18’ 29.8”	6.8	26	1	Loam	Not found
			2	E 098° 28’ 27.2”	6.8	26	1	Loam	Not found
			3		6.8	27	1	Loam	Found
			4	Elev = 502 m	6.8	27	1	Loam	Not found
			5		6.6	27	1	Loam	Not found
16/6/2018	6.06 p.m.	5	1	N 19° 18’ 29.8”	6.8	26	1	Loam	Not found
			2	E 098° 28’ 28.9”	6.6	26	1	Loam	Found
			3		6.8	26	1	Loam	Found
			4	Elev = 507 m	6.8	26	1	Loam	Not found
			5		6.6	26	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	6.11 p.m.	6	1	N 19° 18’ 28.9”	7	26	1	Loam	Not found
			2	E 098° 28’ 28.7”	6.4	28	1	Loam	Not found
			3		6.8	28	1	Loam	Not found
			4	Elev = 509 m	6.8	28	1	Loam	Not found
			5		6.6	28	2	Loam	Not found
16/6/2018	6.16 p.m.	7	1	N 19° 18’ 28.2”	7	27	1	Loam	Not found
			2	E 098° 28’ 30.6”	7	27	1	Loam	Not found
			3		7	27	1	Loam	Not found
			4	Elev = 506 m	7	27	1	Loam	Not found
			5		7	27	1	Loam	Not found
16/6/2018	6.21 p.m.	8	1	N 19° 18’ 29.1”	6.4	27	2	Loam	Not found
			2	E 098° 28’ 30.8”	6.8	27	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 509 m	6.8	27	1	Loam	Found
			5		7	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	6.26 p.m.	9	1	N 19° 18’ 28.0”	6.8	26	1	Loam	Not found
			2	E 098° 28’ 31.6”	6.8	26	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 509 m	6.8	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found
16/6/2018	6.31 p.m.	10	1	N 19° 18’ 28.6”	6.8	27	1	Loam	Not found
			2	E 098° 28’ 31.6”	6.8	27	1	Loam	Not found
			3		6.8	27	1	Loam	Found
			4	Elev = 509 m	6.8	27	1	Loam	Not found
			5		7	26	1	Loam	Not found
16/6/2018	6.36 p.m.	11	1	N 19° 18’ 27.5”	6.8	26	1	Loam	Not found
			2	E 098° 28’ 33.5”	6.8	27	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 519 m	6.8	27	1	Loam	Found
			5		6.8	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	6.41 p.m.	12	1	N 19° 18’ 27.7”	6.8	26	1	Loam	Not found
			2	E 098° 28’ 33.2”	6.8	26	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 517 m	6.6	26	1	Loam	Not found
			5		6.8	27	1	Loam	Not found
16/6/2018	6.46 p.m.	13	1	N 19° 18’ 25.8”	6.8	28	1	Loam	Not found
			2	E 098° 28’ 34.1”	6.6	29	2	Loam	Not found
			3		6.8	29	1	Loam	Not found
			4	Elev = 520 m	6.8	29	2	Loam	Not found
			5		6.8	29	1	Loam	Not found
16/6/2018	6.51 p.m.	14	1	N 19° 18’ 26.5”	6.8	27	1	Loam	Not found
			2	E 098° 28’ 34.1”	6.8	27	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 520 m	6.8	27	1	Loam	Not found
			5		6.8	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	6.56 p.m.	15	1	N 19° 18’ 26.0”	6.6	27	2	Loam	Found
			2	E 098° 28’ 32.1”	6.6	27	2	Loam	Found
			3		6.6	27	1	Loam	Not found
			4	Elev = 528 m	6.6	27	3	Loam	Not found
			5		6.8	27	1	Loam	Not found
16/6/2018	7.01 p.m.	16	1	N 19° 18’ 25.9”	6.6	27	1	Loam	Not found
			2	E 98° 28’ 34.4”	6.4	27	3	Loam	Not found
			3		6.6	27	1	Loam	Not found
			4	Elev = 522 m	6.6	27	2	Loam	Found
			5		6.6	26	1	Loam	Not found
16/6/2018	7.06 p.m.	17	1	N 19° 18’ 27.7”	6.8	28	1	Loam	Found
			2	E 098° 28’ 30.3”	6.6	28	1	Loam	Not found
			3		6.8	28	1	Loam	Not found
			4	Elev = 520 m	6.6	28	1	Loam	Found
			5		6.6	28	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
16/6/2018	7.11 p.m.	18	1	N 19° 18’ 25.3”	6.8	28	1	Loam	Not found
			2	E 098° 28’ 32.5”	6.6	28	1	Loam	Not found
			3		6.6	27	2	Loam	Not found
			4	Elev = 523 m	6.6	27	1	Loam	Not found
			5		6.6	27	2	Loam	Not found
16/6/2018	7.17 p.m.	19	1	N 19° 18’ 28.5”	6.8	28	1	Loam	Not found
			2	E 098° 28’ 29.5”	7	28	1	Loam	Not found
			3		6.6	28	3	Loam	Not found
			4	Elev = 510 m	6.8	28	1	Loam	Not found
			5		6.8	28	1	Loam	Found
16/6/2018	7.20 p.m.	20	1	N 19° 18’ 26.5”	6.8	27	1	Loam	Not found
			2	E 098° 28’ 30.9”	6.8	27	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 522 m	6.8	27	1	Loam	Found
			5		6.8	27	1	Loam	Found

Table 28 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Nan province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	10.50 a.m.	1	1	N 18° 29’ 12.8”	7	32	1	Loam	Not found
			2	E 100° 30’ 30.3”	7	33	1	Loam	Not found
			3		7	32	1	Loam	Not found
			4	Elev = 402 m	7	33	1	Loam	Not found
			5		7	32	1	Loam	Not found
25/10/2019	10.55 a.m.	2	1	N 18° 30’ 04.1”	7	28	1	Gravelly soil	Not found
			2	E 100° 31’ 24.0”	7	28	1	Gravelly soil	Not found
			3		7	28	1	Gravelly soil	Not found
			4	Elev = 372 m	7	28	1	Gravelly soil	Not found
			5		7	28	1	Gravelly soil	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	11.00 a.m.	3	1	N 18° 31’ 25.2”	7	29	1	Loam	Not found
			2	E 100° 34’ 32.1”	7	29	1	Loam	Not found
			3		6.8	29	1	Loam	Not found
			4	Elev = 300 m	7	29	1	Loam	Not found
			5		7	29	1	Loam	Not found
25/10/2019	11.05 a.m.	4	1	N 18° 32’ 13.5”	6.8	28	1.5	Sandy loam	Not found
			2	E 100° 40’ 07.8”	6.8	28	1	Sandy loam	Not found
			3		6.8	30	1.5	Clay	Not found
			4	Elev = 236 m	6.8	28	1.5	Clay	Not found
			5		6.8	29	1	Clay	Not found
25/10/2019	11.10 a.m.	5	1	N 18° 38’ 33.2”	5.4	32	3	Clay	Not found
			2	E 100° 44’ 25.6”	5.2	33	2	Clay	Not found
			3		4	33	8	Clay	Not found
			4	Elev = 196 m	4	32	6	Clay	Not found
			5		4	33	6	Clay	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	11.15 a.m.	6	1	N 18° 38’ 43.0”	7	31	1	Loam	Not found
			2	E 100° 44’ 26.2”	7	31	2	Loam	Not found
			3		7	31	2.5	Loam	Not found
			4	Elev = 197 m	7	31	1	Loam	Not found
			5		7	31	1	Loam	Not found
25/10/2019	11.20 a.m.	7	1	N 18° 38’ 43.5”	5	30	7	Loam	Found
			2	E 100° 44’ 27.5”	6.6	30	1.5	Loam	Found
			3		6.6	30	1	Loam	Not found
			4	Elev = 194 m	6.8	30	1	Loam	Not found
			5		6	30	5.4	Loam	Not found
25/10/2019	11.25 a.m.	8	1	N 18° 38’ 44.0”	6.8	30	2	Loam	Not found
			2	E 100° 44’ 22.5”	6.8	30	1	Loam	Not found
			3		6.7	31	2	Loam	Not found
			4	Elev = 189 m	6.8	31	1	Loam	Not found
			5		6.6	31	2	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	11.30 a.m.	9	1	N 18° 38’ 43.5”	6.9	32	1	Sandy loam	Not found
			2	E 100° 44’ 23.2”	6.6	31	2	Sandy loam	Not found
			3		6.8	31	1	Sandy loam	Not found
			4	Elev = 187 m	5.8	32	4.5	Sandy loam	Not found
			5		6	33	2	Sandy loam	Not found
25/10/2019	11.35 a.m.	10	1	N 18° 39’ 02.6”	7	33	1	Loam	Not found
			2	E 100° 44’ 00.1”	7	33	1	Loam	Not found
			3		7	33	1	Loam	Not found
			4	Elev = 207 m	7	33	1	Loam	Not found
			5		7	32	1	Loam	Not found
25/10/2019	11.40a.m.	11	1	N 18° 39’ 05.6”	7	30	2	Loam	Not found
			2	E 100° 43’ 55.9”	7	30	1	Loam	Not found
			3		7	30	1	Loam	Not found
			4	Elev = 205 m	7	30	1	Loam	Not found
			5		7	30	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	11.45 a.m.	12	1	N 18° 39’ 04.4”	7	31	1	Loam	Not found
			2	E 100° 43’ 57.2”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 205 m	7	30	2	Loam	Not found
			5		6.8	30	3	Loam	Found
25/10/2019	11.50 a.m.	13	1	N 18° 43’ 50.9”	6.8	29	6.5	Sandy loam	Not found
			2	E 100° 45’ 04.5”	7	29	2	Sandy loam	Not found
			3		7	30	1	Sandy loam	Not found
			4	Elev = 201 m	7	30	1	Sandy loam	Not found
			5		7	31	1.5	Sandy loam	Not found
25/10/2019	11.55 a.m.	14	1	N 18° 43’ 59.0”	6.4	29	4.5	Loam	Not found
			2	E 100° 44’ 43.8”	6.4	29	2	Loam	Not found
			3		6.8	29	1.5	Loam	Not found
			4	Elev = 201 m	6.4	30	3	Clay	Not found
			5		4	30	5	Clay	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	12.00 p.m.	15	1	N 18° 43’ 58.7”	4	29	8	Clay	Not found
			2	E 100° 44’ 45.0”	4	29	8	Clay	Not found
			3		4	29	8	Clay	Not found
			4	Elev = 191 m	6.8	29	2	Loam	Not found
			5		6.2	29	1	Loam	Not found
25/10/2019	12.05 p.m.	16	1	N 18° 44’01.7”	6.6	30	1	Loam	Not found
			2	E 100° 44’ 44.6”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 202 m	6.8	29	2	Loam	Not found
			5		6.8	29	1.5	Loam	Not found
25/10/2019	12.10 p.m.	17	1	N 18° 43’ 43.8”	7	34	1	Loam	Not found
			2	E 100° 43’ 10.1”	7	34	1	Loam	Not found
			3		7	34	1	Loam	Not found
			4	Elev = 235 m	7	34	1	Loam	Not found
			5		7	34	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	12.15 p.m.	18	1	N 18° 43’ 44.0”	6.6	34	1.5	Gravelly soil	Not found
			2	E 100° 43’ 12.3”	7	34	1	Gravelly soil	Not found
			3		7	34	1	Gravelly soil	Not found
			4	Elev = 233 m	7	34	1	Gravelly soil	Not found
			5		7	33	1	Gravelly soil	Not found
25/10/2019	12.20 p.m.	19	1	N 18° 43’ 38.6”	6.8	33	2	Loam	Not found
			2	E 100° 42’ 55.9”	6.8	33	1	Loam	Not found
			3		6.6	33	2	Loam	Not found
			4	Elev = 214 m	6.6	33	1	Loam	Not found
			5		6.6	33	1	Loam	Not found
25/10/2019	12.25 p.m.	20	1	N 18° 43’ 37.2”	6	30	1	Clay	Not found
			2	E 100° 42’ 56.1”	6	30	2	Clay	Not found
			3		6	30	7	Clay	Not found
			4	Elev = 214 m	5.4	30	2	Clay	Not found
			5		5.6	30	2	Clay	Not found

Table 29 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Phayao province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	9.35 p.m.	1	1	N 19° 02’ 59.8”	6.4	31	2.5	Loam	Not found
			2	E 099° 58’ 07.7”	6.8	30	2	Loam	Not found
			3		6.8	30	1	Loam	Not found
			4	Elev = 427 m	7	30	1	Loam	Not found
			5		7	31	2	Loam	Not found
25/10/2019	9.40 p.m.	2	1	N 19° 03’ 53.8”	6	30	2	Loam	Not found
			2	E 099° 55’ 43.2”	7	30	1	Loam	Not found
			3		7	30	1	Loam	Not found
			4	Elev = 420 m	7	30	2	Loam	Not found
			5		7	30	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	9.45 p.m.	3	1	N 19° 03’ 54.5”	7	29	1	Loam	Not found
			2	E 099° 55’ 43.0”	7	30	1	Loam	Not found
			3		6.6	30	1	Loam	Not found
			4	Elev = 421 m	7	30	1	Loam	Not found
			5		7	30	1	Loam	Not found
25/10/2019	9.50 p.m.	4	1	N 19° 09’ 17.3”	6.2	37	6	Loam	Not found
			2	E 099° 56’ 45.9”	4.8	37	8	Loam	Not found
			3		7	36	1	Loam	Not found
			4	Elev = 392 m	7	36	5	Loam	Not found
			5		7	35	1	Loam	Not found
25/10/2019	10.00 p.m.	5	1	N 19° 09’ 23.1”	5.6	31	8	Clay	Not found
			2	E 099° 57’ 12.9”	4.8	31	8	Clay	Not found
			3		4.4	31	8	Clay	Not found
			4	Elev = 389 m	4.4	31	8	Clay	Not found
			5		4.4	31	8	Clay	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	10.05 p.m.	6	1	N 19° 11’ 13.4”	5	33	4.5	Clay	Not found
			2	E 100° 01’ 09.0”	3.8	33	8	Clay	Not found
			3		3.8	33	8	Clay	Not found
			4	Elev = 386 m	3.8	33	8	Clay	Not found
			5		5.2	33	8	Clay	Not found
25/10/2019	10.10 p.m.	7	1	N 19° 11’ 13.1”	5.4	33	6	Clay	Not found
			2	E 100° 01’ 09.6”	4.8	33	8	Clay	Not found
			3		3	33	8	Clay	Not found
			4	Elev = 389 m	3.2	33	8	Clay	Not found
			5		3.6	32	8	Clay	Not found
25/10/2019	10.15 p.m.	8	1	N 19° 12’ 49.9”	7	33	2	Gravelly soil	Not found
			2	E 100° 02’ 18.3”	6.8	33	2	Gravelly soil	Not found
			3		7	33	2	Gravelly soil	Not found
			4	Elev = 390 m	6.5	32	5.5	Gravelly soil	Not found
			5		6.2	32	7	Gravelly soil	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	10.20 p.m.	9	1	N 19° 12’ 48.8”	6.6	31	4	Clay loam	Not found
			2	E 100° 02’ 19.4”	6.8	31	4	Clay loam	Not found
			3		5.8	30	7.5	Clay loam	Not found
			4	Elev = 391 m	4	30	8	Clay loam	Not found
			5		6	30	6	Clay loam	Not found
25/10/2019	10.25 p.m.	10	1	N 19° 12’ 48.1”	6.6	31	3.5	Loam	Not found
			2	E 100° 02’ 19.1”	6.6	31	4	Loam	Not found
			3		6.6	31	5	Loam	Not found
			4	Elev = 393 m	6.2	30	5	Loam	Not found
			5		6.6	30	4.5	Loam	Not found

Table 30 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Phetchaburi province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	3.27 p.m.	1	1	N 12° 48’ 37.8”	6.4	24	3.5	Loam	Not found
			2	E 009° 34’ 04.9”	5.2	24	7	Loam	Not found
			3		5.2	24	6	Loam	Found
			4	Elev = 216 m	5.2	24	4	Loam	Not found
			5		5.6	24	4.5	Loam	Not found
10/1/2018	3.33 p.m.	2	1	N 12° 48’ 37.9”	6.4	25	2	Loam	Not found
			2	E 009° 34’ 06.6”	5.8	25	4	Loam	Not found
			3		6.2	25	6	Loam	Not found
			4	Elev = 220 m	5.2	25	3	Loam	Found
			5		6.4	25	3.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	3.35 p.m.	3	1	N 12° 48’ 37.6”	4.4	25	3.5	N12 48 37.6	Not found
			2	E 009° 34’ 09.1”	5	25	3.5	E009 34 09.1	Found
			3		5.6	25	5		Not found
			4	Elev = 212 m	6	25	5	Elev = 212 m	Not found
			5		6.4	25	4.5		Not found
10/1/2018	3.41 p.m.	4	1	N 12° 48’ 37.9”	5.6	24	4.5	N12 48 37.9	Not found
			2	E 009° 34’ 10.5”	5.2	24	3	E009 34 10.5	Not found
			3		6	24	5		Not found
			4	Elev = 218 m	4.4	24	4	Elev = 218 m	Not found
			5		5.5	25	4		Not found
10/1/2018	3.53 p.m.	5	1	N 12° 48’ 45.4”	5	25	8	N12 48 45.4	Not found
			2	E 009° 36’ 25.0”	5.4	25	7	E009 36 25.0	Not found
			3		6.6	25	1.5		Not found
			4	Elev = 101 m	5.4	25	7	Elev = 101 m	Not found
			5		6.6	25	4.5		Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	3.56 p.m.	6	1	N 12° 48’ 45.9”	6.2	25	6	Loam	Not found
			2	E 009° 36’ 27.0”	6.4	24	2	Loam	Found
			3		6.2	25	6	Loam	Not found
			4	Elev = 101 m	5.8	24	5	Loam	Not found
			5		6.8	24	1	Loam	Not found
10/1/2018	4.02 p.m.	7	1	N 12° 48’ 46.8”	6	25	4.5	Loam	Not found
			2	E 009° 36’ 30.9”	6.4	25	2.5	Loam	Not found
			3		6.2	25	7	Loam	Not found
			4	Elev = 101 m	6.2	24	2	Loam	Not found
			5		6.2	25	1.5	Loam	Not found
10/1/2018	4.04 p.m.	8	1	N 12° 48’ 47.0”	6.6	25	1	Loam	Not found
			2	E 009° 36’ 32.4”	5.6	25	5	Loam	Not found
			3		5.8	25	7	Loam	Found
			4	Elev = 99 m	5.6	25	4.5	Loam	Not found
			5		5.6	25	3.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	4.07 p.m.	9	1	N 12° 48’ 47.3”	5.2	24	8	Loam	Not found
			2	E 009° 36’ 34.0”	5.2	24	6	Loam	Not found
			3		5.4	24	6	Loam	Not found
			4	Elev = 99 m	5.8	24	6	Loam	Not found
			5		5.2	24	1	Loam	Not found
10/1/2018	4.09 p.m.	10	1	N 12° 48’ 48.2”	5	24	7.5	Loam	Not found
			2	E 009° 36’ 37.7”	6.2	24	5	Loam	Found
			3		5.2	24	6.5	Loam	Not found
			4	Elev = 100 m	5	24	5.5	Loam	Found
			5		6	24	1	Clay loam	Not found
10/1/2018	4.15 p.m.	11	1	N 12° 54’ 35.4”	4.8	25	9.5	Loam	Found
			2	E 009° 39’ 26.7”	5.2	25	7.5	Loam	Not found
			3		5.4	25	4.5	Loam	Not found
			4	Elev = 69 m	5.2	25	2.5	Loam	Not found
			5		5.2	25	6	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	4.20 p.m.	12	1	N 12° 54’ 36.5”	4.8	25	6	Loam	Not found
			2	E 009° 39’ 26.9”	5.4	25	4	Loam	Not found
			3		5.6	25	4.5	Loam	Not found
			4	Elev = 67 m	6.2	25	2.5	Clay	Not found
			5		5.8	25	4	Clay	Not found
10/1/2018	4.25 p.m.	13	1	N 12° 54’ 34.6”	5.6	25	7	Loam	Not found
			2	E 009° 39’ 27.6”	4.2	25	7	Loam	Not found
			3		5.4	25	6	Loam	Not found
			4	Elev = 66 m	5.4	25	7	Loam	Not found
			5		5.4	25	4	Loam	Not found
10/1/2018	4.30 p.m.	14	1	N 12° 54’ 38.0”	6.4	25	6	Loam	Not found
			2	E 009° 39’ 26.9”	4.8	25	2	Loam	Not found
			3		6	25	2	Loam	Not found
			4	Elev = 67 m	5.6	25	5	Loam	Not found
			5		5.2	25	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	4.35 p.m.	15	1	N 12° 54’ 35.3”	5.5	26	3	Loam	Not found
			2	E 009° 39’ 27.7”	5.4	25	3	Loam	Not found
			3		6	26	2	Loam	Not found
			4	Elev = 66 m	5.4	26	4	Loam	Not found
			5		5	26	2.5	Loam	Not found
10/1/2018	4.40 p.m.	16	1	N 12° 54’ 35.4”	5.8	25	3.5	Loam	Not found
			2	E 009° 39’ 28.2”	6.4	25	1.5	Loam	Not found
			3		6	25	4.5	Loam	Not found
			4	Elev = 67 m	6.2	25	2.5	Loam	Not found
			5		5	25	5	Loam	Not found
10/1/2018	4.45 p.m.	17	1	N 12° 54’ 35.9”	5.4	25	6.5	Loam	Not found
			2	E 009° 39’ 28.6”	5.6	25	7	Loam	Not found
			3		5.8	25	6	Loam	Not found
			4	Elev = 71 m	5	25	7	Loam	Not found
			5		4.8	25	7	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/1/2018	5.38 p.m.	18	1	N 12° 55’ 59.3”	6.4	25	2.5	Loam	Not found
			2	E 009° 49’ 21.5”	6.4	25	3	Loam	Not found
			3		6.4	25	2	Loam	Not found
			4	Elev = 16 m	5	25	5.5	Loam	Not found
			5		6	25	5.5	Loam	Not found
10/1/2018	5.45 p.m.	19	1	N 12° 55’ 58.9”	5	25	2.5	Clay	Not found
			2	E 009° 49’ 21.8”	5.2	25	1	Clay	Not found
			3		5.2	25	5	Clay	Not found
			4	Elev = 21 m	5	25	1.5	Clay	Not found
			5		5	25	1.5	Clay	Found
10/1/2018	5.50 p.m.	20	1	N 12° 55’ 59.8”	6.2	25	1.5	Loam	Not found
			2	E 009° 49’ 21.9”	5	25	4	Loam	Not found
			3		6.2	25	2.5	Loam	Found
			4	Elev = 21 m	6.2	25	2.5	Loam	Not found
			5		6.4	25	3.5	Loam	Not found

Table 31 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Phrae province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
24/10/2019	9.45 a.m.	1	1	N 18° 21’ 33.5”	7	32	1	Loam	Not found
			2	E 100° 20’ 37.4”	7	32	1	Loam	Not found
			3		7	30	1	Loam	Not found
			4	Elev = 217 m	7	32	1	Loam	Not found
			5		7	32	1	Loam	Not found
24/10/2019	9.50 a.m.	2	1	N 18° 25’ 01.2”	6.6	29	1	Loam	Not found
			2	E 100° 28’ 34.7”	6.8	29	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 442 m	6.8	29	1	Loam	Not found
			5		6.6	29	1.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
24/10/2019	9.55 a.m.	3	1	N 18° 25’ 01.8”	6.8	29	1	Loam	Not found
			2	E 100° 28’ 34.3”	6.8	28	1	Loam	Not found
			3		6.6	29	2	Loam	Not found
			4	Elev = 421 m	6.5	29	1	Loam	Not found
			5		6.8	30	2	Loam	Not found
25/10/2019	9.00 a.m.	4	1	N 18° 25’ 31.4”	6.6	29	2	Sandy loam	Not found
			2	E 100° 10’ 33.8”	4.2	29	8	Sandy loam	Not found
			3		5.2	29	8	Sandy loam	Not found
			4	Elev = 185 m	5	29	7	Sandy loam	Not found
			5		5	28	7	Sandy loam	Not found
25/10/2019	9.05 a.m.	5	1	N 18° 25’ 29.6”	4.2	29	8	Sandy loam	Not found
			2	E 100° 10’ 35.1”	4.4	29	8	Sandy loam	Not found
			3		4.6	29	8	Sandy loam	Not found
			4	Elev = 208 m	4.2	29	8	Sandy loam	Not found
			5		4.8	29	8	Sandy loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	9.10 a.m.	6	1	N 18° 26’ 53.0”	5.8	28	5	Loam	Not found
			2	E 100° 09’ 09.1”	4.4	30	6	Loam	Not found
			3		5.6	30	8	Loam	Found
			4	Elev = 192 m	6	30	4	Loam	Not found
			5		4.8	29	7	Loam	Not found
25/10/2019	9.15 a.m.	7	1	N 18° 26’ 53.2”	4.4	29	6	Loam	Not found
			2	E 100° 09’ 10.3”	5.8	29	5	Loam	Not found
			3		4.8	28	4	Loam	Not found
			4	Elev = 185 m	4.4	28	6	Sandy loam	Not found
			5		5	28	6	Loam	Not found
25/10/2019	9.20 p.m.	8	1	N 18° 27’ 16.9”	6.2	29	2	Loam	Not found
			2	E 100° 08’ 37.8”	6.2	29	2	Loam	Not found
			3		6.2	29	2	Loam	Not found
			4	Elev = 190 m	6.2	29	2	Loam	Not found
			5		6.2	29	4	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
25/10/2019	9.25 p.m.	9	1	N 18° 27’ 16.5”	6.2	29	4	Loam	Not found
			2	E 100° 08’ 38.3”	6	29	5	Loam	Not found
			3		5.2	28	4	Loam	Not found
			4	Elev = 193 m	6	28	6	Loam	Not found
			5		6.4	28	3	Loam	Not found
25/10/2019	9.30 p.m.	10	1	N 18° 27’ 16.1”	6.2	30	2	Loam	Not found
			2	E 100° 08’ 38.9”	5.6	30	6	Loam	Not found
			3		7.2	30	1	Gravelly soil	Not found
			4	Elev = 194 m	5.2	29	7	Clay loam	Not found
			5		5	30	4	Loam	Not found

Table 32 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Sakon Nakhon province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	8.50 a.m.	1	1	N 17° 07’ 03.05”	7	26	1	Loam	Not found
			2	E 104° 01’ 43.2”	6.8	26	1	Loam	Not found
			3		7	26	1	Loam	Not found
			4	Elev = 220 m	6.8	26	1	Loam	Not found
			5		7	26	1	Loam	Not found
3/6/2018	8.56 a.m.	2	1	N 17° 07’ 04.0”	6.8	26	1	Loam	Not found
			2	E 104° 01’ 41.7”	6.8	28	1	Loam	Not found
			3		6.8	28	1	Loam	Not found
			4	Elev = 218 m	6.8	27	1	Loam	Not found
			5		6.8	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	9.01 a.m.	3	1	N 17° 06’ 57.4”	6.8	26	1	Loam	Not found
			2	E 104° 01’ 22.8”	6.8	26	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 267 m	6.8	27	1	Loam	Not found
			5		6.8	27	1	Loam	Found
3/6/2018	9.05 a.m.	4	1	N 17° 06’ 58.6”	6.8	26	1	Loam	Not found
			2	E 104° 00’ 21.9”	6.8	27	1	Loam	Not found
			3		6.8	26	1	Loam	Not found
			4	Elev = 261 m	6.8	27	1	Loam	Not found
			5		6.8	27	1	Loam	Not found
3/6/2018	9.08 a.m.	5	1	N 17° 06’ 55.9”	7	26	1	Sandy	Not found
			2	E 104° 00’ 28.3”	7	26	1	Sandy loam	Not found
			3		7	26	1	Sandy loam	Not found
			4	Elev = 364 m	7	26	1	Sandy loam	Not found
			5		7	26	1	Sandy loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	9.11a.m.	6	1	N 17° 06’ 56.2”	7	26	1	Gravelly soil	Not found
			2	E 104° 00’ 24.8”	7	26	1	Loam	Not found
			3		7	26	1	Loam	Not found
			4	Elev = 326 m	7	26	1	Gravelly soil	Not found
			5		7	26	1	Gravelly soil	Not found
3/6/2018	9.21 a.m.	7	1	N 17° 06’ 11.4”	6	27	1	Loam	Not found
			2	E 103° 59’ 41.5”	6	27	1	Loam	Found
			3		6	27	1	Loam	Found
			4	Elev = 359 m	6.8	25	1	Loam	Found
			5		7	25	1	Loam	Not found
3/6/2018	9.23 a.m.	8	1	N 17° 06’ 11.2”	6.8	26	1	Loam	Found
			2	E 103° 59’ 8.6”	6.8	26	1	Loam	Not found
			3		6.8	26	2	Loam	Not found
			4	Elev = 348 m	6.6	26	1	Loam	Not found
			5		6.6	26	2	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	9.30 a.m.	9	1	N 17° 05’ 23.7”	6.8	27	1	Loam	Found
			2	E 103° 59’ 8.6”	6.8	27	2	Loam	Not found
			3		6.7	26	1	Loam	Found
			4	Elev = 330 m	6.8	27	1	Loam	Found
			5		7	26	1	Loam	Not found
3/6/2018	9.39 a.m.	10	1	N 17° 05’ 12.3”	6.8	27	1	Loam	Found
			2	E 103° 59’ 11.8”	6.8	28	1	Loam	Not found
			3		6.8	25	1	Loam	Not found
			4	Elev = 327 m	6.8	25	1	Loam	Not found
			5		6.8	25	1	Loam	Not found
3/6/2018	9.48 a.m.	11	1	N 17° 01’ 38.6”	7	29	1	Loam	Not found
			2	E 103° 58’ 28.2”	6.8	27	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 301 m	6.4	27	1	Loam	Found
			5		6.4	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	9.49 a.m.	12	1	N 17° 01’ 37.7”	6.8	28	1	Loam	Not found
			2	E 103° 58’ 27.7”	6.8	28	1	Loam	Not found
			3		7	28	1	Loam	Found
			4	Elev = 298 m	6.8	28	1	Loam	Not found
			5		6.6	28	3	Loam	Not found
3/6/2018	10.26 a.m.	13	1	N 16° 50’ 50.5”	7	27	2	Loam	Not found
			2	E 103° 55’ 16.8”	7	28	1	Loam	Not found
			3		7	27	1	Loam	Not found
			4	Elev = 312 m	7	27	1	Loam	Not found
			5		7	26	1	Loam	Not found
3/6/2018	10.30 a.m.	14	1	N 16° 50’ 49.7”	6.8	26	1	Loam	Not found
			2	E 103° 55’ 18.1”	7	26	1	Loam	Not found
			3		7	27	1	Loam	Not found
			4	Elev = 315 m	7	27	1	Loam	Not found
			5		7	27	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	10.34 a.m.	15	1	N 16° 50’ 41.1”	7	29	1	Loam	Not found
			2	E 103° 54’ 56.7”	6.8	28	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 343 m	6.8	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found
3/6/2018	10.44 a.m.	16	1	N 16° 50’ 07.7”	7	28	1	Loam	Found
			2	E 103° 54’ 19.2”	6.8	29	1	Loam	Found
			3		6.8	28	1	Loam	Found
			4	Elev = 413 m	6.8	27	1	Loam	Found
			5		6.8	27	1	Loam	Found
3/6/2018	10.53 a.m.	17	1	N 16° 49’ 02.4”	7	27	1	Loam	Not found
			2	E 103° 53’ 41.4”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 507 m	7	27	1	Loam	Not found
			5		6.8	26	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	10.55 a.m.	18	1	N 16° 49’ 02.4”	6.8	27	1	Loam	Not found
			2	E 103° 53’ 40.0”	7	27	1	Loam	Not found
			3		6.8	27	1	Loam	Not found
			4	Elev = 506 m	7	26	1	Loam	Not found
			5		6.8	26	1	Loam	Not found
3/6/2018	11.01 a.m.	19	1	N 16° 48’ 45.3”	7	28	1	Loam	Not found
			2	E 103° 53’ 28.8”	7	27	1	Loam	Not found
			3		7	27	1	Loam	Not found
			4	Elev = 555 m	6.8	28	1	Loam	Not found
			5		7	27	1	Loam	Not found
3/6/2018	11.10 a.m.	20	1	N 16° 48’ 41.3”	6.8	26	1	Loam	Found
			2	E 103° 53’ 22.2”	6.6	26	1	Loam	Found
			3		6.6	25	2	Loam	Found
			4	Elev = 530 m	6.2	26	2	Loam	Found
			5		6.6	26	2	Loam	Not found

Table 33 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Khon Kaen province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	3.48 p.m.	1	1	N 16° 16’ 37.3”	7	28	1	Loam	Not found
			2	E 102° 47’ 01.0”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 178 m	6.8	28	1	Loam	Not found
			5		7	29	1	Loam	Found
3/6/2018	3.57 p.m.	2	1	N 16° 16’ 38.1”	7	28	1	Loam	Found
			2	E 102° 47’ 02.0”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 171 m	7	28	1	Loam	Found
			5		7	28	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	4.02 p.m.	3	1	N 16° 15’ 12.9”	6.6	28	1	Sandy loam	Found
			2	E 102° 46’ 29.0”	7	29	1	Loam	Not found
			3		6.2	29	1	Loam	Not found
			4	Elev = 168 m	6.8	28	1	Loam	Not found
			5		6.8	29	1	Loam	Not found
3/6/2018	4.07 p.m.	4	1	N 16° 15’ 13.9”	6.8	29	1	Loam	Found
			2	E 102° 46’ 29.5”	6.8	28	1	Loam	Found
			3		6.7	28	1	Loam	Not found
			4	Elev = 168 m	6.8	28	1	Loam	Not found
			5		6.8	29	1	Loam	Not found
3/6/2018	4.10 p.m.	5	1	N 16° 15’ 42.3”	7	30	1	Loam	Not found
			2	E 102° 46’ 38.4”	7	30	1	Loam	Not found
			3		7	29	1	Loam	Found
			4	Elev = 165 m	7	30	1	Loam	Not found
			5		7	30	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	4.15 p.m.	6	1	N 16° 15’ 41.3”	7	29	1	Loam	Not found
			2	E 102° 46’ 37.7”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 166 m	7	29	1	Loam	Not found
			5		7	28	1	Loam	Not found
3/6/2018	4.20 p.m.	7	1	N 16° 16’ 19.2”	6.8	28	1	Loam	Not found
			2	E 102° 46’ 52.7”	6.8	29	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 166 m	6.8	29	1	Loam	Not found
			5		6.8	29	1	Loam	Not found
3/6/2018	4.25 p.m.	8	1	N 16° 16’ 18.1”	6.8	28	1	Loam	Not found
			2	E 102° 46’ 52.3”	6.8	28	1	Loam	Found
			3		7	28	1	Loam	Found
			4	Elev = 167 m	7	28	1	Loam	Not found
			5		6.8	28	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
3/6/2018	4.30 p.m.	9	1	N 16° 16’ 46.2”	6.8	28	1	Loam	Not found
			2	E 102° 47’ 03.4”	6.8	28	1	Loam	Not found
			3		6.8	28	1	Loam	Not found
			4	Elev = 165 m	7	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found
3/6/2018	4.35 p.m.	10	1	N 16° 16’ 47.0”	6.8	28	1	Loam	Found
			2	E 102° 47’ 03.2”	6.8	28	1	Loam	Not found
			3		7	28	1	Loam	Found
			4	Elev = 163 m	7	28	1	Loam	Found
			5		7	28	1	Loam	Found

Table 34 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Saraburi province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	10.30 a.m.	1	1	N 14° 40’ 220”	7	28	1	Loam	Not found
			2	E 100° 53’ 3”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 60 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found
10/6/2018	10.31 a.m.	2	1	N 14° 40’ 24”	7	28	1	Loam	Not found
			2	E 100° 53’ 3”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 70 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	10.42 a.m.	3	1	N 14° 40’ 30.1”	7	28	1	Loam	Not found
			2	E 100° 52’ 57.5”	6.8	29	1	Loam	Not found
			3		6.6	29	1	Loam	Not found
			4	Elev = 58 m	6.8	28	1	Loam	Not found
			5		7	28	1	Loam	Found
10/6/2018	10.41 a.m.	4	1	N 14° 40’ 29.7”	7	28	1	Loam	Not found
			2	E 100° 52’ 58.4”	6.8	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 60 m	7	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found
10/6/2018	10.49 a.m.	5	1	N 14° 40’ 39.8”	7	29	1	Gravelly soil	Not found
			2	E 100° 52” 46.3”	7	29	1	Gravelly soil	Not found
			3		7	28	1	Gravelly soil	Not found
			4	Elev = 81 m	7	29	1	Gravelly soil	Not found
			5		7	28	1	Gravelly soil	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	10.51 a.m.	6	1	N 14° 40’ 39.4”	7	29	1	Gravelly soil	Found
			2	E 100° 52’ 47.1”	7	29	1	Gravelly soil	Not found
			3		7	29	1	Loam	Found
			4	Elev = 72 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found
10/6/2018	10.57 a.m.	7	1	N 14° 40’ 44.5”	7	28	1	Loam	Not found
			2	E 100° 52’ 37.2”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 70 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found
10/6/2018	11.00 a.m.	8	1	N 14° 40’ 44.3”	7	28	1	Loam	Not found
			2	E 100° 52’ 36.6”	7	28	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 72 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	11.10 a.m.	9	1	N 14° 40’ 40.2”	7	28	1	Loam	Not found
			2	E 100° 52’ 48.6”	7	27	1	Loam	Not found
			3		7	28	1	Loam	Not found
			4	Elev = 85 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found
10/6/2018	11.15 a.m.	10	1	N 14° 40’ 40.7”	7	29	1	Loam	Found
			2	E 100° 52’ 49.1”	7	29	1	Loam	Found
			3		7	29	1	Loam	Found
			4	Elev = 84 m	7	29	1	Loam	Not found
			5		7	29	1	Loam	Not found
10/6/2018	11.20 a.m.	11	1	N 14° 40’ 36.5”	7	28	1	Loam	Not found
			2	E 100° 52’ 59.4”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 89 m	7	28	1	Loam	Not found
			5		7	28	1	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	11.23 a.m.	12	1	N 14° 40’ 36.0”	7	28	1	Loam	Not found
			2	E 100° 53’ 00.4”	7	29	1	Loam	Not found
			3		7	29	1	Loam	Not found
			4	Elev = 77 m	7	29	1	Loam	Found
			5		7	29	1	Loam	Not found
10/6/2018	1.25 p.m.	13	1	N 14° 26’ 28.8”	6.8	28	1	Loam	Found
			2	E 100° 57’ 39.1”	6.8	28	1	Loam	Not found
			3		6.8	28	2	Loam	Found
			4	Elev = 47 m	6.8	28	1	Loam	Not found
			5		6.8	28	1	Loam	Not found
10/6/2018	1.27 p.m.	14	1	N 14° 26’ 29.2”	7	28	1	Loam	Found
			2	E 100° 57’ 38.1”	6.8	28	1	Loam	Found
			3		6.8	28	1	Loam	Not found
			4	Elev = 37 m	6.8	28	1	Loam	Not found
			5		6.8	29	1	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	1.32 p.m.	15	1	N 14° 26’ 27.0”	6.6	28	1	Gravelly soil	Not found
			2	E 100° 57’ 37.5”	6.4	29	1	Gravelly soil	Not found
			3		6.6	29	1	Gravelly soil	Not found
			4	Elev = 50 m	6.8	28	1	Gravelly soil	Not found
			5		7	28	1	Gravelly soil	Not found
10/6/2018	1.37 p.m.	16	1	N 14° 26’ 26.4”	6.8	29	1	Sandy loam	Not found
			2	E 100° 57’ 39.4”	6.8	28	1	Loam	Not found
			3		6.8	28	1	Sandy loam	Not found
			4	Elev = 46 m	6.8	28	1	Loam	Found
			5		7	29	1	Loam	Not found
10/6/2018	1.42 p.m.	17	1	N 14° 26’ 17.0”	5.2	25	2	Loam	Not found
			2	E 100° 57’ 43.0”	5.4	26	2	Loam	Not found
			3		6.2	26	3	Loam	Not found
			4	Elev = 60 m	5	26	3	Loam	Not found
			5		6	26	2	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
10/6/2018	1.47 p.m.	18	1	N 14° 26’ 19.0”	6.8	25	1	Loam	Not found
			2	E 100° 57’ 41.0”	6.4	25	2	Loam	Not found
			3		6.2	25	2	Loam	Found
			4	Elev = 60 m	6.6	25	3	Loam	Not found
			5		6.2	26	4	Loam	Not found
10/6/2018	1.52 p.m.	19	1	N 14° 26’ 17.0”	6.4	25	2	Loam	Found
			2	E 100° 57’ 43.0”	6.2	25	1	Loam	Not found
			3		6.4	25	1	Loam	Not found
			4	Elev = 60 m	6.2	25	1	Loam	Not found
			5		6.2	25	1	Loam	Found
10/6/2018	1.58 p.m.	20	1	N 14° 26’ 22.0”	5	25	3	Loam	Not found
			2	E 100° 57’ 40.0”	5.8	25	4	Loam	Not found
			3		6	25	3	Loam	Not found
			4	Elev = 70 m	6.4	25	4	Loam	Not found
			5		5	26	6	Loam	Not found

Table 35 Extrinsic factors, geographic coordinates and findings of EPNs in soil samples from Uttaradit province (No. = number, GPS = Global Positioning System, Elev = Elevation)

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	10.50 a.m.	1	1	N 17° 42’ 28.8”	5.8	24	2.5	Loam	Not found
			2	E 100° 56’ 50.9”	5.6	24	7.0	Loam	Not found
			3		5.0	24	6.0	Loam	Not found
			4	Elev = 641 m	5.2	24	5.0	Loam	Not found
			5		5.8	24	6.5	Loam	Not found
23/10/2017	10.55 a.m.	2	1	N 17° 42’ 27.9”	6.8	23	1.5	Loam	Not found
			2	E 100° 56’ 51.0”	6.2	23	5.5	Loam	Not found
			3		6.8	24	2.0	Loam	Not found
			4	Elev = 687 m	6.0	24	6.5	Clay loam	Not found
			5		6.6	24	2.5	Loam	Found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	11.00 a.m.	3	1	N 17° 42’ 31.4”	4.8	23	8.0	Clay loam	Not found
			2	E 100° 56’ 51.6”	4.6	24	8.0	Loam	Not found
			3		5.4	23	6.5	Loam	Not found
			4	Elev = 700m	5.0	25	7.0	Loam	Not found
			5		4.8	25	8.0	Loam	Not found
23/10/2017	11.05 a.m.	4	1	N 17° 42’ 29.7”	6.0	25	6.5	Loam	Not found
			2	E 100° 56’ 50.9”	5.8	24	4.5	Loam	Not found
			3		6.2	25	6.5	Loam	Not found
			4	Elev = 677 m	5.0	24	8.0	Loam	Not found
			5		6.4	25	5.0	Loam	Not found
23/10/2017	11.10 a.m.	5	1	N 17° 42’ 32.9”	5.0	25	6.0	Loam	Not found
			2	E 100° 56’ 52.5”	5.2	26	8.0	Loam	Not found
			3		7.2	26	8.0	Loam	Not found
			4	Elev = 703 m	4.6	27	8.0	Loam	Not found
			5		4.4	27	5.0	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	11.15 a.m.	6	1	N 17° 42’ 32.1”	6.4	24	5.0	Loam	Not found
			2	E 100° 56’ 53.2”	5.0	25	8.0	Loam	Not found
			3		5.4	25	7.0	Loam	Not found
			4	Elev = 699 m	5.2	24	8.0	Loam	Not found
			5		6.6	24	4.0	Loam	Not found
23/10/2017	11.20 a.m.	7	1	N 17° 42’ 32.3”	5.4	25	7.0	Clay loam	Not found
			2	E 100° 56’ 48.8”	6.0	24	5.5	Clay loam	Not found
			3		5.1	24	8.0	Clay loam	Not found
			4	Elev = 714 m	5.2	25	8.0	Clay loam	Not found
			5		4.8	26	8.0	Clay loam	Not found
23/10/2017	11.25 a.m.	8	1	N 17° 43’ 46.9”	5.4	23	8.0	Clay loam	Not found
			2	E 100° 56’ 50.2”	5.2	23	4.0	Clay loam	Not found
			3		5.2	25	6.5	Clay loam	Not found
			4	Elev = 777 m	5.2	25	8.0	Clay loam	Not found
			5		5.4	24	3.5	Clay loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	11.30 a.m.	9	1	N 17° 43’ 34.3”	6.6	24	3.0	Clay loam	Not found
			2	E 100° 56’ 48.5”	6.2	25	6.0	Clay loam	Not found
			3		6.4	25	4.0	Clay loam	Not found
			4	Elev = 697 m	5.0	25	7.0	Clay loam	Not found
			5		4.6	23	7.5	Clay loam	Not found
23/10/2017	11.35 a.m.	10	1	N 17° 44’ 23.6”	4.8	23	8.0	Loam	Not found
			2	E 100° 56’ 52.4”	6.0	23	4.0	Loam	Found
			3		4.4	23	8.0	Loam	Not found
			4	Elev = 673 m	5.0	20	8.0	Loam	Not found
			5		4.4	23	8.0	Loam	Not found
23/10/2017	11.40 a.m.	11	1	N 17° 43’ 477”	5.0	23	7.5	Clay loam	Not found
			2	E 100° 56’ 50.4”	5.2	24	7.5	Clay loam	Not found
			3		5.2	24	8.0	Clay loam	Not found
			4	Elev = 743 m	4.4	24	7.5	Clay loam	Not found
			5		4.4	24	8.0	Clay loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	11.45 a.m.	12	1	N 17° 44’ 36.1”	6.0	24	5.5	Loam	Not found
			2	E 100° 56’ 51.1”	5.2	24	7.0	Loam	Not found
			3		4.6	24	8.0	Loam	Not found
			4	Elev = 655 m	4.8	24	8.0	Loam	Not found
			5		5.8	24	2.0	Loam	Not found
23/10/2017	11.50 a.m.	13	1	N 17° 44’ 23.6”	6.2	24	4.0	Loam	Not found
			2	E 100° 56’ 52.4”	6.4	23	5.5	Loam	Not found
			3		6.4	23	4.5	Loam	Not found
			4	Elev = 673 m	6.6	23	2.0	Loam	Not found
			5		6.8	23	3.0	Loam	Not found
23/10/2017	11.55 a.m.	14	1	N 17° 45’ 19.4”	2.0	27	6.4	Loam	Not found
			2	E 100° 56’ 26.7”	6.0	26	3.0	Loam	Not found
			3		6.0	27	6.0	Loam	Not found
			4	Elev = 547 m	5.8	26	4.5	Loam	Not found
			5		6.4	26	1.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	12.00 p.m.	15	1	N 1°7 44’ 36.1”	5.4	24	7.5	Clay loam	Not found
			2	E 100° 56’ 51.1”	5.2	24	8.0	Clay loam	Found
			3		5.4	24	4.5	Clay loam	Not found
			4	Elev = 682 m	6.4	24	1.5	Clay loam	Not found
			5		5.2	24	7.0	Clay loam	Not found
23/10/2017	12.05 p.m.	16	1	N 17° 44’ 09.7”	6.2	26	1.5	Clay loam	Not found
			2	E 100° 56’ 53.1”	6.4	26	2.0	Clay loam	Not found
			3		5.6	27	4.0	Clay loam	Not found
			4	Elev = 715 m	6.0	26	6.5	Clay loam	Not found
			5		5.2	27	8.0	Loam	Not found
23/10/2017	12.10 p.m.	17	1	N 17° 45’ 19.0”	6.0	26	4.5	Loam	Not found
			2	E 10°0 56’ 27.2”	5.2	26	4.5	Loam	Not found
			3		5.2	27	8.0	Loam	Found
			4	Elev = 545 m	6.2	27	2.0	Loam	Not found
			5		6.2	26	3.0	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	12.15 p.m.	18	1	N 17° 44’ 09.4”	6.4	26	2.0	Clay loam	Not found
			2	E 100° 56’ 52.7”	6.2	26	2.0	Clay loam	Found
			3		6.8	26	5.5	Clay loam	Not found
			4	Elev = 714 m	5.6	26	2.5	Clay loam	Not found
			5		5.8	26	6.0	Clay loam	Not found
23/10/2017	12.20 p.m.	19	1	N 17° 43’ 58.7”	5.0	23	8.0	Loam	Not found
			2	E 100° 56’ 53.7”	4.8	23	7.5	Loam	Not found
			3		5.2	24	8.0	Loam	Not found
			4	Elev = 728 m	6.2	24	4.0	Loam	Not found
			5		4.8	24	6.0	Loam	Not found
23/10/2017	12.25 p.m.	20	1	N 17° 43’ 58.9”	5.4	24	6.5	Loam	Not found
			2	E 100° 56’ 53.0”	6.2	24	5.0	Loam	Not found
			3		5.4	24	6.0	Loam	Not found
			4	Elev =727 m	5.0	26	5.5	Loam	Not found
			5		4.8	23	7.0	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	12.30 p.m.	21	1	N 17° 43’ 39.9”	5.0	26	4.5	Loam	Not found
			2	E 100° 56’ 51.1”	6.0	26	4.5	Loam	Not found
			3		5.8	26	2.5	Clay loam	Not found
			4	Elev = 737 m	5.4	25	3.0	Clay loam	Not found
			5		5.0	25	8.0	Clay loam	Not found
23/10/2017	12.35 p.m.	22	1	N 17° 43’ 39.7”	6.0	25	8.5	Clay loam	Not found
			2	E 100° 56’ 52.2”	5.8	25	3.5	Loam	Not found
			3		6.1	25	2.5	Loam	Not found
			4	Elev = 730 m	6.0	25	5.0	Loam	Not found
			5		6.0	25	8.0	Loam	Not found
23/10/2017	12.40 p.m.	23	1	N 17° 43’ 32.6”	5.6	23	5.5	Loam	Not found
			2	E 100° 56’ 52.7”	6.0	23	7.0	Loam	Not found
			3		5.0	23	6.0	Clay loam	Not found
			4	Elev = 753 m	5.2	23	7.5	Loam	Not found
			5		6.0	23	6.0	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	12.45 p.m.	24	1	N 17° 43’ 33.0”	5.6	23	5.0	Loam	Not found
			2	E 100° 56’ 52.7”	5.0	23	8.0	Loam	Not found
			3		5.4	24	7.0	Loam	Not found
			4	Elev = 753 m	6.0	23	6.5	Loam	Not found
			5		5.2	24	4.5	Loam	Not found
23/10/2017	12.50 p.m.	25	1	N 17° 43’ 20.5”	6.0	24	8.0	Loam	Not found
			2	E 100° 56’ 49.2”	5.4	24	7.5	Loam	Not found
			3		5.6	24	8.0	Loam	Not found
			4	Elev = 763 m	6.2	24	7.0	Loam	Not found
			5		5.2	24	7.5	Loam	Not found
23/10/2017	12.55 p.m.	26	1	N 17° 43’ 21.0”	6.4	25	6.5	Loam	Not found
			2	E 100° 56’ 49.0”	5.0	24	8.0	Loam	Not found
			3		5.6	24	7.5	Loam	Not found
			4	Elev = 753 m	5.4	24	1.0	Loam	Not found
			5		5.6	24	6.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	1.00 p.m.	27	1	N 17° 43’ 09.0”	5.4	24	7.5	Loam	Not found
			2	E 100° 56’ 48.3”	6.0	24	6.5	Loam	Not found
			3		5.6	24	7.5	Loam	Found
			4	Elev = 776 m	5.2	24	7.5	Loam	Not found
			5		6.0	24	6.5	Loam	Not found
23/10/2017	1.05 p.m.	28	1	N 17° 43’ 09.0”	6.8	24	5.0	Loam	Not found
			2	E 100° 56’ 48.2”	5.2	25	8.0	Loam	Found
			3		5.4	24	6.5	Loam	Not found
			4	Elev = 785 m	4.8	25	7.5	Loam	Not found
			5		5.4	23	6.5	Loam	Found
23/10/2017	1.10 p.m.	29	1	N 17° 43’ 08.0”	6.5	24	2.0	Loam	Not found
			2	E 100° 56’ 52.3”	0.2	24	8.0	Loam	Not found
			3		6.2	24	7.5	Loam	Not found
			4	Elev = 782 m	6.2	24	6.0	Loam	Not found
			5		5.2	24	7.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	1.15 p.m.	30	1	N 17° 43’ 080”	6.2	25	7.0	Loam	Not found
			2	E 100° 56’ 51.8”	6.0	24	6.5	Loam	Not found
			3		6.8	24	6.5	Loam	Not found
			4	Elev = 781 m	6.0	24	7.0	Loam	Not found
			5		5.8	24	7.0	Loam	Not found
23/10/2017	1.20 p.m.	31	1	N 17° 42’ 58.2”	4.8	25	6.0	Clay loam	Not found
			2	E 100° 56’ 49.9”	5.0	25	8.0	Clay loam	Not found
			3		6.8	27	1.5	Clay loam	Not found
			4	Elev = 741 m	6.6	26	1.5	Clay loam	Not found
			5		6.8	26	2.0	Clay loam	Not found
23/10/2017	1.25 p.m.	32	1	N 17° 42’ 59.1”	6.0	25	5.5	Clay loam	Not found
			2	E 100° 56’ 50.3”	5.4	24	7.5	Clay loam	Not found
			3		6.0	25	6.0	Clay loam	Not found
			4	Elev = 749 m	6.2	25	6.5	Clay loam	Not found
			5		6.4	24	4.0	Clay loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	1.30 p.m.	33	1	N 17° 42’ 37.4”	6.4	25	6.0	Clay loam	Not found
			2	E 100° 56’ 50.9”	6.2	25	3.0	Clay loam	Not found
			3		6.2	25	3.5	Clay loam	Not found
			4	Elev = 714 m	6.4	25	5.5	Clay loam	Not found
			5		5.8	25	5.5	Clay loam	Not found
23/10/2017	1.35 p.m.	34	1	N 17° 42’ 38.0”	6.2	27	5.5	Clay loam	Not found
			2	E 100° 56’ 50.4”	6.4	27	2.5	Clay loam	Found
			3		6.6	26	1.5	Clay loam	Not found
			4	Elev = 743 m	5.4	25	4.5	Clay loam	Not found
			5		6.0	28	5.5	Clay loam	Found
23/10/2017	1.40 p.m.	35	1	N 17° 42’ 16.3”	6.6	25	0.0	Loam	Not found
			2	E 100° 56’ 56.6”	6.2	28	5.5	Loam	Not found
			3		6.4	25	4.0	Loam	Not found
			4	Elev = 677 m	6.0	25	2.0	Loam	Not found
			5		6.0	25	5.5	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	1.45 p.m.	36	1	N 17° 42’ 16.2”	6.4	27	3.5	Loam	Not found
			2	E 100° 56’ 57.3”	5.8	26	2.5	Loam	Not found
			3		6.6	25	1.0	Loam	Not found
			4	Elev =679 m	6.2	25	3.2	Loam	Not found
			5		6.2	25	4.0	Loam	Not found
23/10/2017	1.50 p.m.	37	1	N 17° 42’ 06.7”	5.4	29	7.0	Loam	Not found
			2	E 100° 56’ 55.5”	5.4	28	7.5	Loam	Not found
			3		6.0	28	5.0	Loam	Not found
			4	Elev = 684 m	6.2	28	4.0	Loam	Not found
			5		6.2	29	7.0	Loam	Not found
23/10/2017	1.55 p.m.	38	1	N 17° 42’ 06.6”	6.0	28	5.0	Loam	Not found
			2	E 100 °56’ 55.1”	6.4	27	3.0	Loam	Not found
			3		6.2	27	2.0	Loam	Not found
			4	Elev = 685 m	5.8	26	4.5	Loam	Not found
			5		6.2	27	4.0	Loam	Not found

Date (D/M/Y)	Time	Site No.	Sample No.	GPS location	pH	Temperature (°C)	Moisture	Soil type	Findings of EPNs
23/10/2017	2.00 p.m.	39	1	N 17° 41’ 59.2”	6.6	25	1.5	Loam	Not found
			2	E 100° 56’ 41.5”	6.8	25	1.5	Loam	Not found
			3		6.0	25	4.5	Loam	Not found
			4	Elev = 668 m	6.0	25	1.5	Loam	Found
			5		6.6	25	2.5	Loam	Not found
23/10/2017	2.05 p.m.	40	1	N 17° 41’ 55.1”	5.8	25	5.0	Loam	Found
			2	E 100° 56’ 41.2”	5.6	25	4.0	Loam	Not found
			3		6.0	25	5.5	Loam	Not found
			4	Elev = 659 m	6.4	25	4.5	Loam	Not found
			5		6.4	25	2.5	Loam	Not found